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INTRACELLULAR FILAMENT BUNDLES IN WHOLE MOUNTS OF CHICK AND HUMAN MYOBLASTS EXTRACTED WITH TRITON X-100

ABSTRACT. The method of Triton X-100 extraction and critical point drying of whole mounts of cultured chick and human myoblasts was used to study the presence of intracellular bundles of filaments within these cells. Observation by means of transmission and scanning electron microscopy demonstrated a complex system of filament bundles which appeared morphologically and spatially heterogeneous. Most obvious were long dense bundles or cables traversing along the ventral surface of developing myoblasts, presumably the 'stress fibers' seen in light microscopy. Other bundle types occurred which were composed of loose aggregates of filaments coursing through the remnant cell body. A prominent accumulation of filaments was also seen at the lateral edges of these myoblasts. These lateral edge cables were thicker and denser than any other type of filament bundle observed in the myoblasts. Reaction of unextracted myoblasts directly to human antiplatelet myosin conjugated to rhodamine demonstrated that the most intense reaction also occurred along the lateral edges of both human and chick myoblasts. During development of chick myoblasts the filament bundles became oriented parallel to the cell axis giving the cell a fusiform morphology. It is possible that the various filament bundle structures and their differing structural and spatial dispositions could be related to functional differences among the diverse population of intracellular bundles of filaments.

Introduction

A CHARACTERISTIC feature of many cells grown in culture is the ability to adhere to, and spread upon, the underlying substrate. Associated with these capabilities is the formation of intracellular structures termed 'stress fibers' (Lewis and Lewis, 1924). Cells which do not possess spreading properties contain few or no demonstrable 'stress fibers' (Goldman and Follet, 1969). Also, cells which normally anchor and spread out on a substrate can become detached when exposed to proteases with a subsequent loss of 'stress fibers'; re-attachment of these cells with concomitant spreading leads to the reappearance of 'stress fibers' (Goldman, 1975).

The presence of 'stress fibers' in cultured cells has been intensively studied. Phase contrast (Buckley and Porter, 1967; Goldman, 1971) and Nomarski microscopy (Goldman *et al.*, 1975) have demonstrated that these fibers normally occur in the ventral aspect of the cell, along the cortical area of cell–substrate contact. Examination of cultured cells using transmission electron microscopy has also shown that organized parallel arrays of microfilaments occur in these regions which were correlated with the 'stress fibers' observed using light microscopic techniques (Taylor, 1966; McNutt *et al.*, 1971; Goldman, 1971; Luduena and Wessels, 1973; Goldman *et al.*, 1975). 'Stress fibers' were further characterized when it was demonstrated that these bundles of microfilaments reacted with heavy meromyosin (Luduena and Wessels, 1973; Spooner *et al.*, 1973) indicating they contained actin (Ishikawa *et al.*, 1969). With the development of specific antibodies to muscle proteins, various immunofluorescent studies indicated that these

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fibers apart from containing actin (Lazarides and Weber, 1974; Lazarides, 1976; Osborn and Weber, 1977) could be closely associated with myosin, tropomyosin, and alpha-actinin (Weber and Groeschel-Stewart, 1974; Lazarides and Burridge, 1975; Lazarides, 1976). The close proximity of these muscle proteins with 'stress fibers' suggested that these fibrous structures were possible contractile elements involved in motility of cultured cells.

Morphologically, however, 'stress fibers' have not been adequately characterized in their three-dimensional spatial relationships. Previous work (Pudney and Singer, 1979) demonstrated that the use of Triton X-100 extracted, critical-point dried cultured chick myoblasts provided a convenient technique for studying the general pattern of filamentous organization within these cells. Removal of most of the ground cytoplasm and organelles by treatment with detergent left a residuum of filaments plus the nucleus which was preserved in its three-dimensional integrity by critical-point drying (Anderson, 1951). The present study examines the morphology of the 'stress fibers' in extracted chick and human myoblasts and extends previous work by investigating morphological changes in these structures preceding differentiation (fusion) of chick myoblasts in culture. In addition, diversity in the protein composition of bundles of filaments was revealed by immunofluorescence microscopy on chick and human cells, indicating that structural differences among the intracellular fibers may imply functional variability.

Materials and Methods

Chick myoblasts

Cells were prepared from 11-day-old chick embryo breast (pectoralis) muscle by treatment with 0·5% trypsin (Worthington Biochemical, Freehold, N.J.) in Eagle's minimum essential medium (MEM) minus calcium and magnesium, with constant shaking for 20 min at 37°C. The resulting cell suspension was decanted to separate single cells from the tissue and then centrifuged at 700 *g* for 3 min. The resulting cell pellet was suspended in Eagle's MEM supplemented with 10% horse serum and 1% chick embryo extract (supernatant of 11-day-old decapitated chick embryos diluted 1:1 with MEM and centrifuged at 1000 *g* for 10 min to remove cell debris). In order to remove most of the contaminating fibroblasts, cells were plated onto uncoated tissue culture dishes and fibroblasts allowed to attach for 20 min (Yaffe, 1968). The supernatant cell suspension enriched in presumptive myoblasts was then transferred to collagen-coated culture dishes and incubated for specified times.

Human myoblasts

Muscle tissue from human biopsy was finely minced and placed in a culture dish containing MEM plus 20% fetal calf serum. Cells were cultured for a period of 3 weeks, with the medium being changed every week. After 3 weeks, cells had grown sufficiently for subpassage and were detached by means of a solution of trypsin (0·5%) and EDTA (5 mM) in MEM and plated at a density of 10^5 per plate.

Preparation of cells for transmission electron microscopy

Titanium grids (E. Fullam, Inc., Schnectady, N.Y.) were placed on a 5% Formvar film which had been released from a clean slide onto distilled water. The film plus the grids was picked up on a glass coverslip, dried, and lightly coated with carbon. They were then sterilized by immersion in 70% ethanol, dried and placed in a standard Falcon plastic tissue culture dish.

Cultured cells were first washed with MEM and then a solution of 0·05% trypsin and 5 mM EDTA in MEM minus calcium and magnesium for 5 min to remove them from the culture dish. The cell suspension was centrifuged at 700 *g* for 3 min and resuspended in MEM plus 10% horse serum and 1% embryo extract. A suspension of myoblasts was then plated onto the grids in approximately five drops of medium and allowed to incubate for 2 hr. More medium was then added so as to cover the grids and the dishes gently agitated to remove unattached cells from the grids. The incubation was continued for 24 hr, at which time the cells on the grids were viewed under a phase-contrast microscope to determine their condition. The coverslips carrying the grids were removed from the medium, washed in hypotonic buffer (10 mM NaCl, 1·5 mM $MgCl_2$, 10 mM Tris HCl pH 7·4) and placed

in 1% solution of Triton X-100 in the same buffer for 2 min at room temperature. This was followed by gently rinsing in the buffer and then the cells were fixed by immersing the preparations in 5% glutaraldehyde in 0·2 M S-collidine buffer, pH 7·3 for 10 min. The specimens were then briefly washed in 0·2 M S-collidine buffer, followed by distilled water and 30% acetone.

The specimens were then treated with a saturated solution of uranyl acetate in 50% acetone for 3 min. (Subsequent observations of unstained cells demonstrated that there was sufficient inherent contrast in the preparations that staining with uranyl acetate was not necessary.) Dehydration was continued by passing them through a graded series of acetones, 70–100% (2 min each).

Finally, the grids were carefully removed from the coverslips and critical-point dried from CO_2 in a critical-point drier (Polaron Instrument, PA). Following critical-point drying, the cells were lightly coated with carbon (to provide stability in the electron microscope) and observed in a JEOL 100S electron microscope, at either 80 or 100 kV. Stereoscopic pairs were taken at either $\pm 5^\circ$ or $\pm 10^\circ$ from the original axis. Presumptive myoblasts were also cultured for 24 hr in plastic culture dishes and processed exactly as above, except that following fixation the cells were rapidly dehydrated through a graded series of alcohols and embedded in Epon. Thin sections were cut using a diamond knife on a Sorvall Porter-Blum Mark 2 ultramicrotome and contrast was enhanced in these sections by staining in a saturated solution of uranyl acetate in 50% acetone followed by lead citrate (Venable and Coggeshall, 1965). Thin sections were examined in a JEOL 100S electron microscope.

Preparation of cells for scanning electron microscopy

For scanning electron microscopy, cells were prepared by plating them onto culture dishes containing 22 mm round glass coverslips coated with carbon. After culturing for 24 hr, the glass coverslips bearing cultured myoblasts were removed and washed in phosphate buffered saline (PBS). The coverslips were then treated in the same manner as whole mount cells for transmission electron microscopy, except the staining step was omitted and the coverslips were critical-point dried from CO_2 in a Samdri PVT-3 apparatus (Biodynamic Research, Rockville, MD).

The glass coverslips were mounted on aluminum stubs and coated with gold–palladium in a Hummer II sputter-coating apparatus (Technic, Alexandria, VA). They were then viewed in an ETEC Autoscan microscope (Hayward, CA) operated at 20 kV.

Preparation of cells for immunofluorescent microscopy

Cells were removed from culture dishes, plated onto 22 mm round glass coverslips coated with 0·5 mg/ml collagen solution (Worthington Biochemical, Freehold, N.J.) and allowed to grow for desired times. The coverslips were then gently rinsed in PBS and fixed in 2% formaldehyde in PBS for 10 min. This was followed by washing in PBS and treatment with acetone for 20 min at -20°C. After the cells were air-dried, they were incubated with 50 μl of human anti-platelet myosin conjugated to rhodamine for 20 min at room temperature. They were then rinsed with PBS, inverted over a drop of 50% glycerol on a glass slide and the edge of the coverglass sealed with cosmetic nail varnish. Observations were carried out using a Leitz microscope fitted with epifluorescent optics and photomicrographs taken with a Zeiss planapoachromat 63 X oil-immersion objective.

Preparation of antibody

The human antiplatelet myosin was a generous gift from K. Fujiwara, and the preparation and specificity of this antibody has been previously described (Fujiwara and Pollard, 1976).

Results

Chick myoblast cultures

When cultured, chick presumptive myoblasts initially attach and spread upon the substratum to form large, irregular cells. Extraction of these cells with Triton X-100 followed by critical point drying revealed an intricate system of filamentous structures, plus the nucleus (Fig. 1). The gross morphological appearance of the myoblasts is reflected in the spatial arrangement of numerous filamentous bundles, presumably 'stress fibers' which traverse the cell in a seemingly un-

organized manner (Fig. 1). Closer inspection of these bundles of filaments during this initial period of attachment also demonstrated that they were morphologically heterogeneous (Fig. 2). Many filament bundles are very compact and hence appear denser than others which have a looser organization of filaments. These latter bundles could be seen to be composed of microfilaments. Very often these less compact bundles become splayed with the filaments joining part of the microfilamentous reticulum. The diameter of the filamentous bundles also varied greatly from 30 nm for the smallest internal bundle diameter, up to 250 nm for the largest bundle diameter. A distinct bundle of filaments also occurred along the lateral edges of the extracted chick myoblasts (Fig. 1). This accumulation of filaments was so tightly packed as to form dense lateral edge cables delineating the remnant filamentous residuum. These dense lateral edge cables also varied greatly in diameter, from 130 up to 420 nm. This large variation in size was often due to the branching of these cables into smaller filament bundles which then coursed internally.

Dense peripheral concentrations of filaments did not appear to be prominent along all cellular margins. Within a small region, one edge of the cell may have dense lateral edge cables and the other may lack them completely (Fig. 3). At the ends of myoblasts where many pseudopodia and microprocesses were being formed, the dense lateral edge cables are noticeably reduced as they approach this region of the cell (Fig. 4). At the cellular extremities many bundles of filaments became splayed losing their tightly packed configuration. In some instances, however, organized bundles of filaments extended beyond the cellular filamentous reticulum (Fig. 4).

Three-dimensional information obtained from stereoscopic observation showed that the bundles of filaments coursed through a filamentous reticulum composed mainly of 7–8 nm filaments (Fig. 5). These stereomicrographs demonstrated that the large bundles of microfilaments coursed mainly along the cell/substratum region. Many smaller bundles, however, also occurred within the filamentous reticulum of these cells.

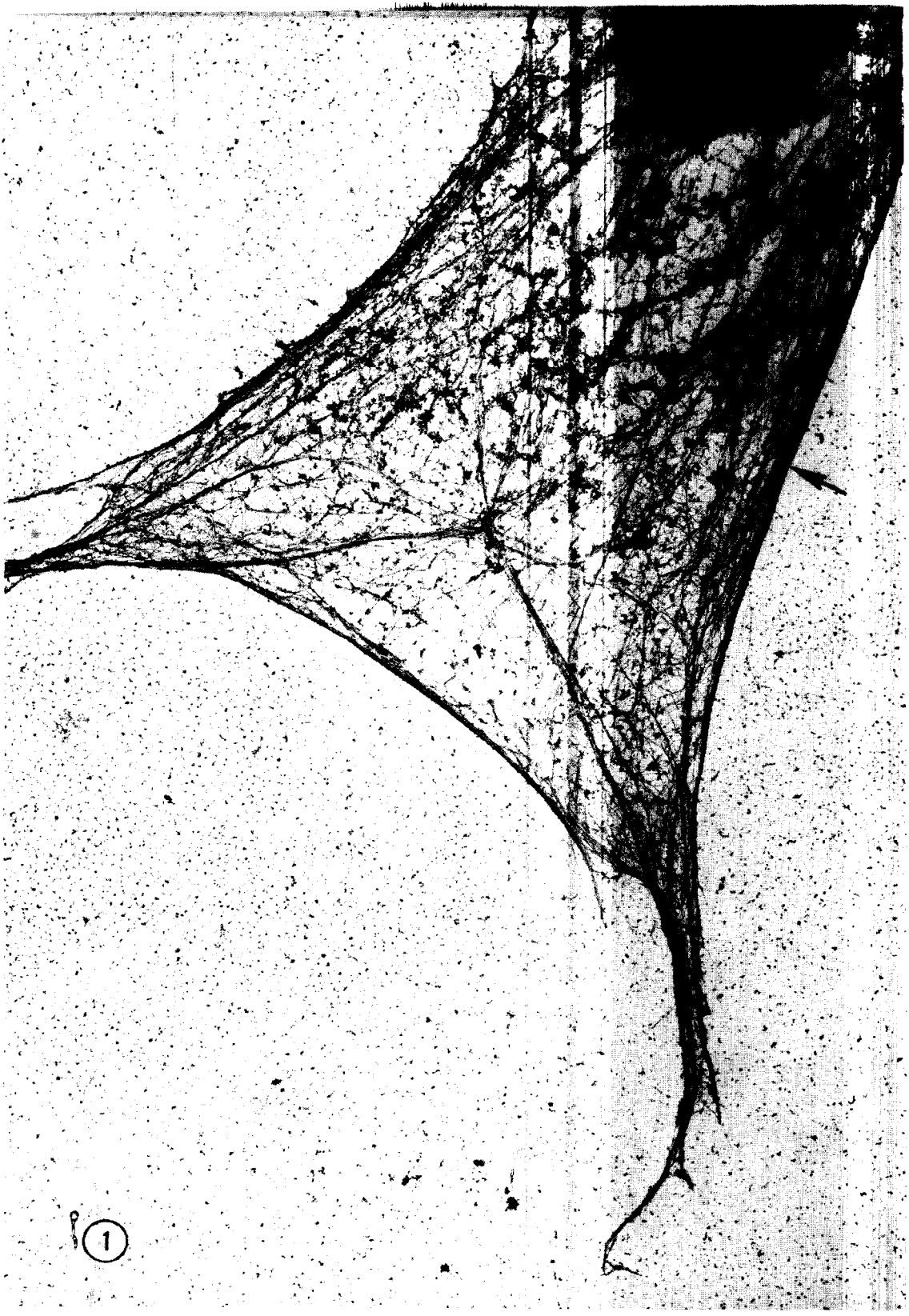
Following a 24 hr culture period most of the presumptive chick myoblasts have become elongated. This change in shape, with the development of a longitudinal axis, is characteristic for normal myoblast development (Huang *et al.*, 1978). This morphological change imposed a polarity upon the myoblasts which once again was reflected in

Fig. 1. This low power micrograph of a presumptive chick myoblast extracted with Triton X-100 demonstrates that this treatment removes most of the cytoplasm and organelles leaving behind an intricate system of filaments plus the nucleus. The complex organization of bundles of filaments is readily apparent. Particularly noticeable is a dense cable of filaments which demarcs the lateral edge of this cell (arrow). × 5600.

Fig. 2. At higher magnification bundles of filaments of varying structural complexity can be seen. Some appear to be very dense (large arrow) while others are composed of less tightly packed filaments (small arrow). The bundles of filaments course either longitudinally, or obliquely to the cell axis, often branching into smaller bundles to form a complex organization. A fine filamentous reticulum is also present in these cells. × 20,800.

Fig. 3. The dense lateral edge cable of this extracted presumptive chick myoblast is only present on one side of the cell (arrow). The other laterial edge lacks this structure. × 15,000.

Fig. 4. At the extremities of presumptive chick myoblasts, numerous cellular projections are developed. This micrograph shows that in these areas the dense lateral edge cables disappear or become less prominent. Also bundles of filaments can be seen to run, uninterrupted, into many of these cellular projections. × 8400.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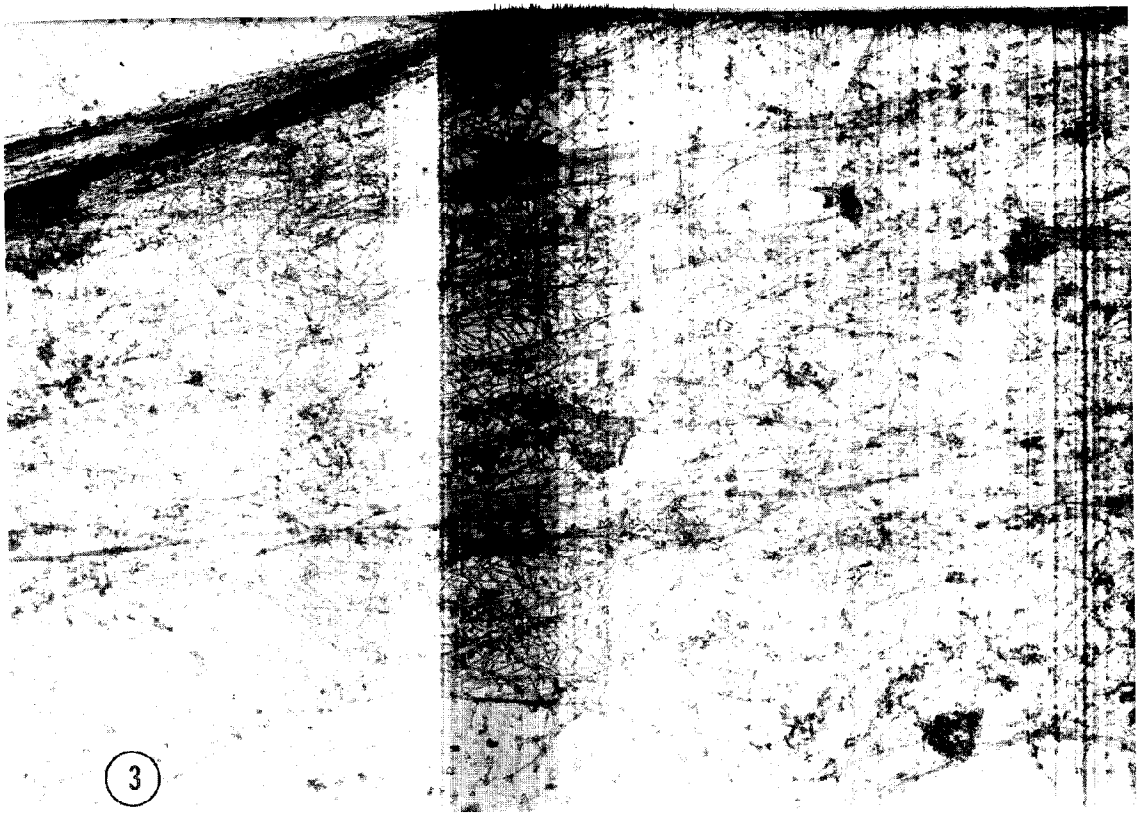
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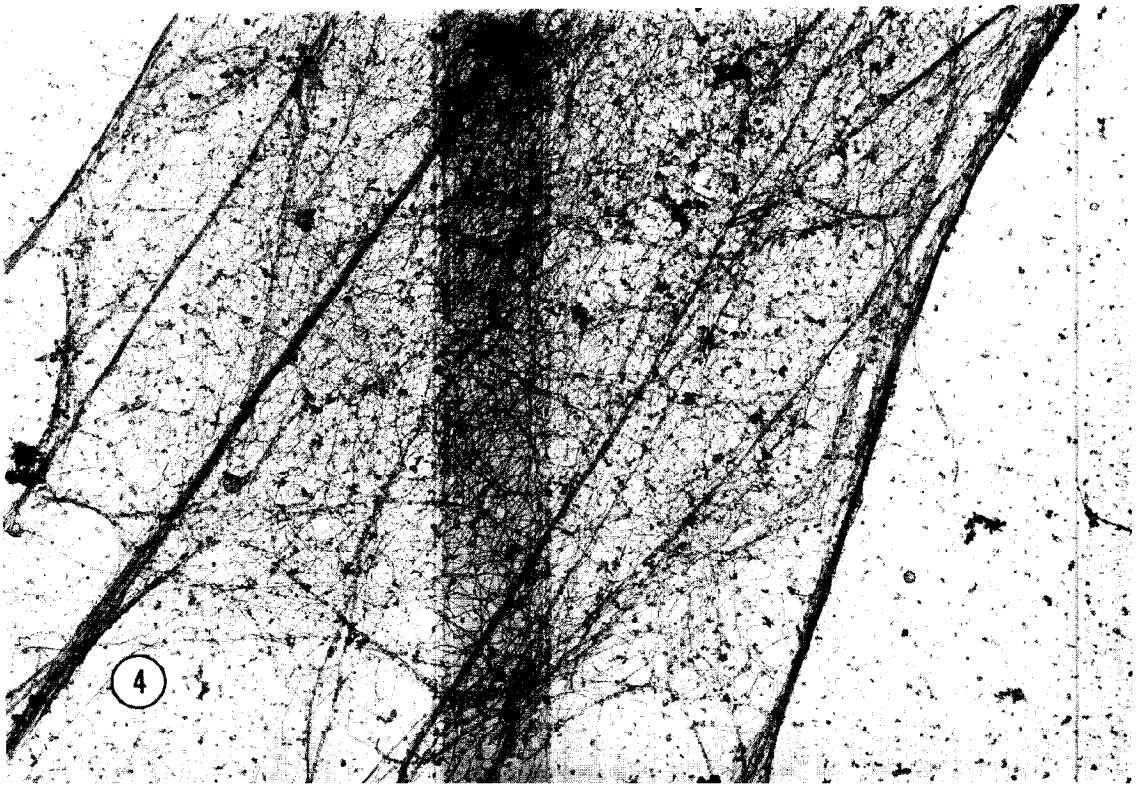
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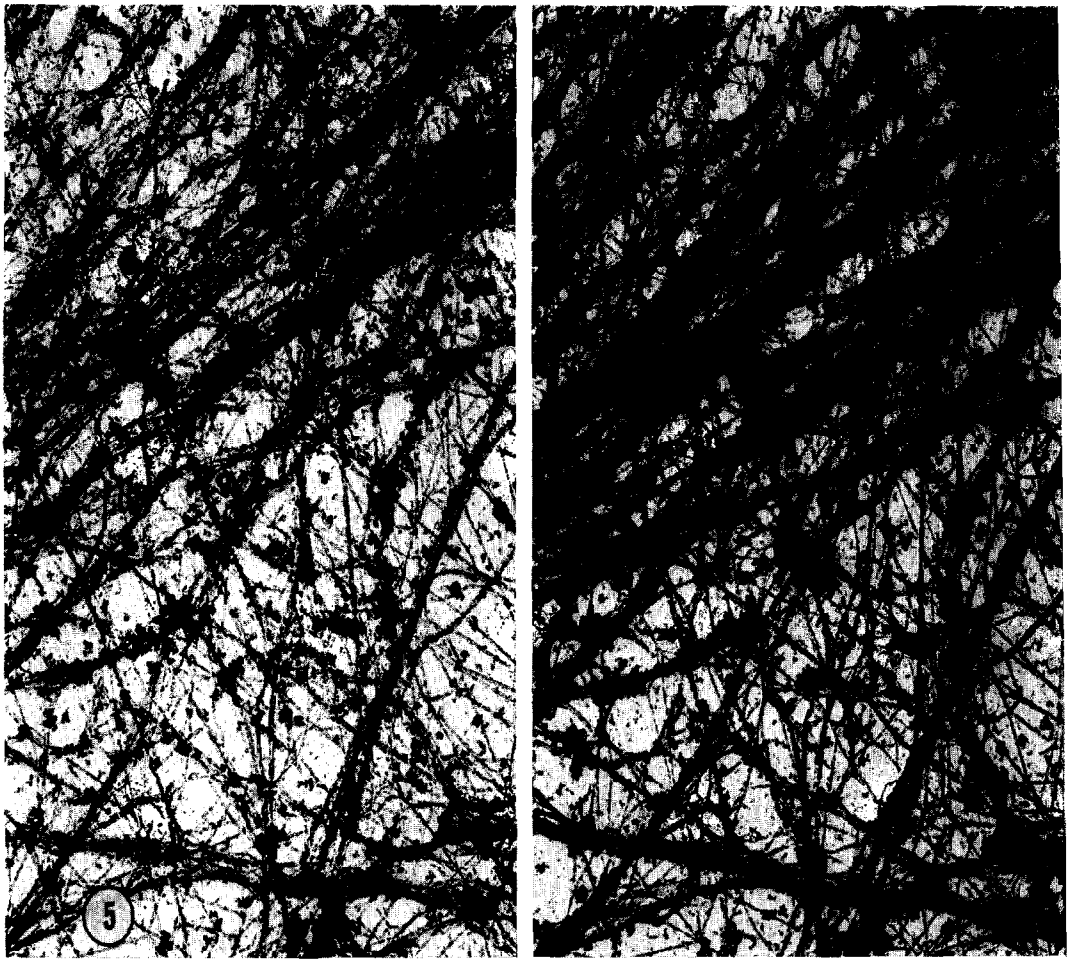
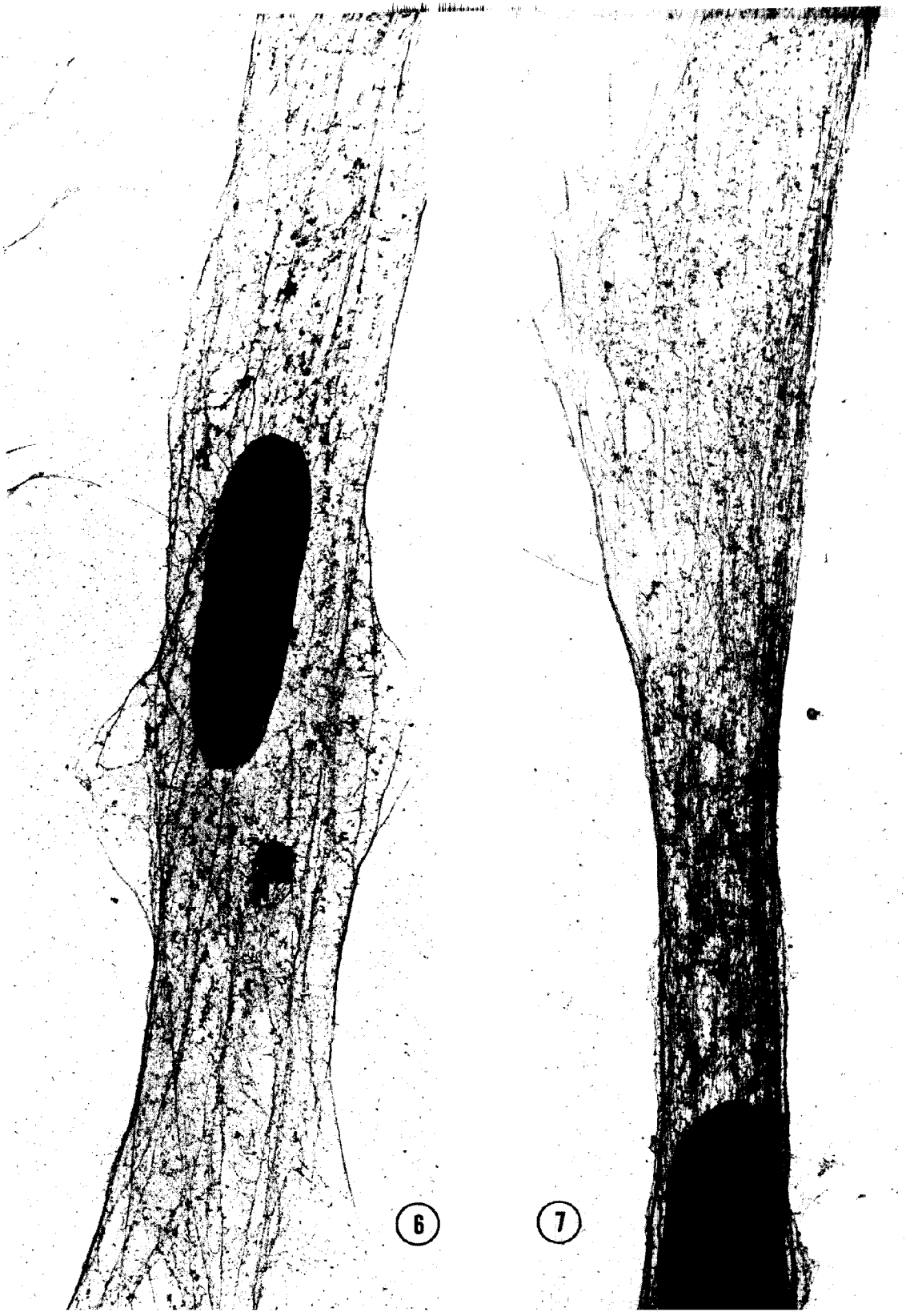


Figs. 5. Stereo pair taken $\pm 10°$ from the original axis. Large bundles of filaments run along the cell/substratum area. Smaller bundles of filaments are present within the filamentous reticulum of the cell. The three-dimensional nature of the filamentous reticulum can also be seen in these micrographs. $\times 24{,}000$.

Figs. 6, 7. Following a 24 hr cultured period the presumptive chick myoblasts begin to elongate to form fusiform cells. At this time most of the intracellular bundles of filaments become reorganized and course parallel to the longitudinal axis of the cell. Fig. 6, $\times 2800$; Fig. 7, $\times 8400$.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the organization of the intracellular bundles of filaments. During this period of differentiation the diverse bundles of filaments had become constrained and are now observed as numerous dense structures coursing longitudinally to the cell axis (Figs. 6, 7). Further development of myoblasts involves the fusion of these cells into syncitial myotubes. Fig. 8 illustrates three myoblasts which are in physical contact, possibly preparatory to fusion into a myotube. Bundles of filaments appear to enter the remnant cell bodies of adjacent myoblasts to possibly establish morphological continuity between the different filamentous matrices (Fig. 8). These filamentous interconnections are presumably the substructural remains of the microprocesses which initiate cellular fusion (Huang *et al.*, 1978).

Thin plastic sections of Triton X-100 extracted chick presumptive myoblasts were also examined in the electron microscope. These observations showed that the 'stress fibers' contained microfilaments and occasionally numerous microtubules. These microtubules were approximately 30 nm in diameter and occurred either singly in these cells or as distinct bundles which often appeared close to the nucleus (Fig. 10) or at the lateral margins of myoblasts (Figs. 9, 10).

Human muscle cultures

Human muscle biopsy tissue, when cultured, produced extremely large well-spread cells. These cells also contained many bundles of filaments which became extremely prominent when the cells were treated with Triton X-100 (Fig. 11). The bundles were also structurally heterogeneous, varying from dense compact configurations to loose aggregates of filaments. They also formed complex arrays and were seen to branch as well as anastomose with neighboring bundles. As in chick myoblasts, these bundles coursed through and were often connected to a diffuse three-dimensional reticulum of 7–8 nm filaments. The human myoblasts also displayed dense aggregations of filaments along the lateral edges of the cells (Fig. 12). These aggregations, as in chick myoblasts, were much larger in diameter (approximately 300 nm) and more tightly packed than other filament bundles. At high magnification part of the morphology of these lateral edge cables was revealed, and large diameter (approximately 40–50 nm) structures were seen to be present (Fig. 13).

Scanning electron microscopy

Observations on myoblasts extracted with Triton X-100 using scanning electron micro-

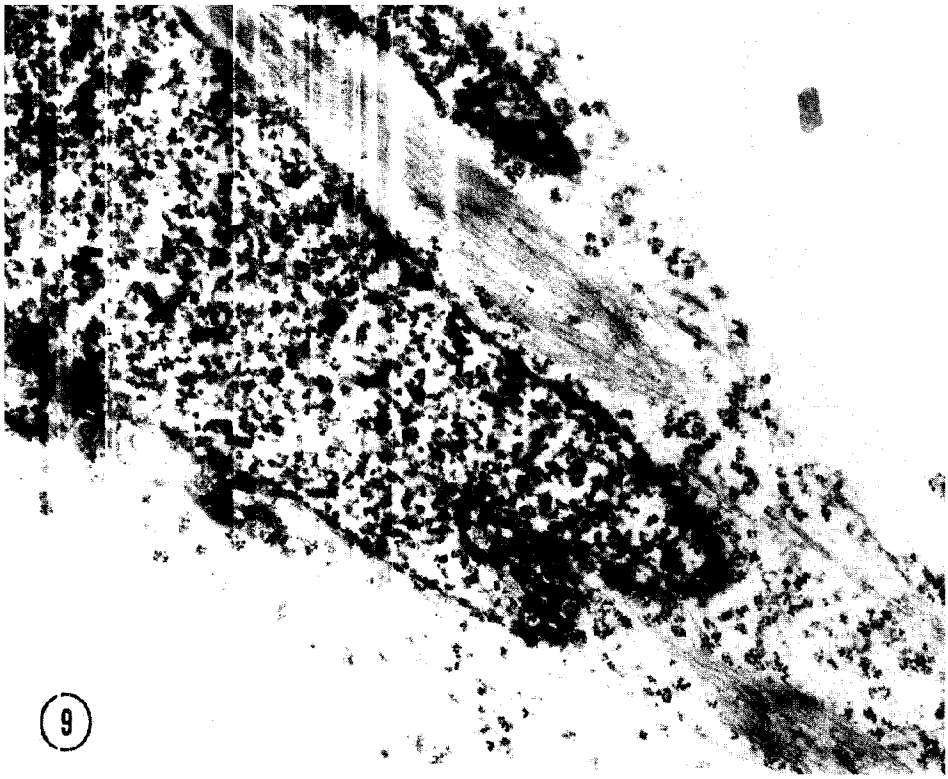
Fig. 8. Three chick myoblasts which appear to be in physical contact, possibly prior to fusion. The arrows point to points of filament continuity between these cells. × 8100.

Figs. 9, 10. Presumptive chick myoblasts extracted with Triton X-100 were also embedded in plastic. Thin sectioned material demonstrated that numerous microtubules were present which often formed large bundles. These bundles of microtubules were frequently seen in close association with the nucleus (Fig. 9) or aligned along the lateral edges of the cell (Fig. 10). Fig. 9, × 30,000; Fig. 10, × 40,000.

Fig. 11. Part of a human myoblast demonstrating the presence of bundles of filaments in these cells following extraction with Triton X-100. × 10,160.

Fig. 12. High-powered micrograph of the lateral edge cable of an extracted human myoblast. It can be seen that this large structure (approximately 300 nm diameter) is so dense that it is difficult to determine its composition. × 47,800.

Fig. 13. In this micrograph an area of the dense lateral edge cable can be seen. It is composed, in part, of large structures (approximately 40–50 nm in diameter). Closely associated with these cables is the filamentous reticulum, made up of filaments 7–8 nm (small arrows) and 10 nm (arrowhead) in diameter. × 106,400.



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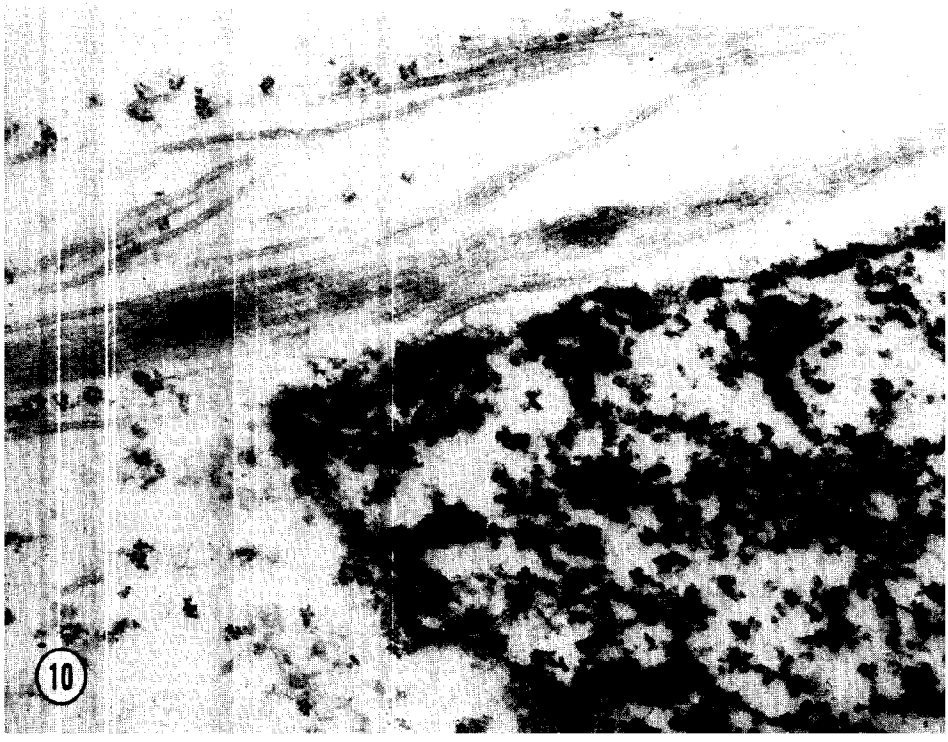

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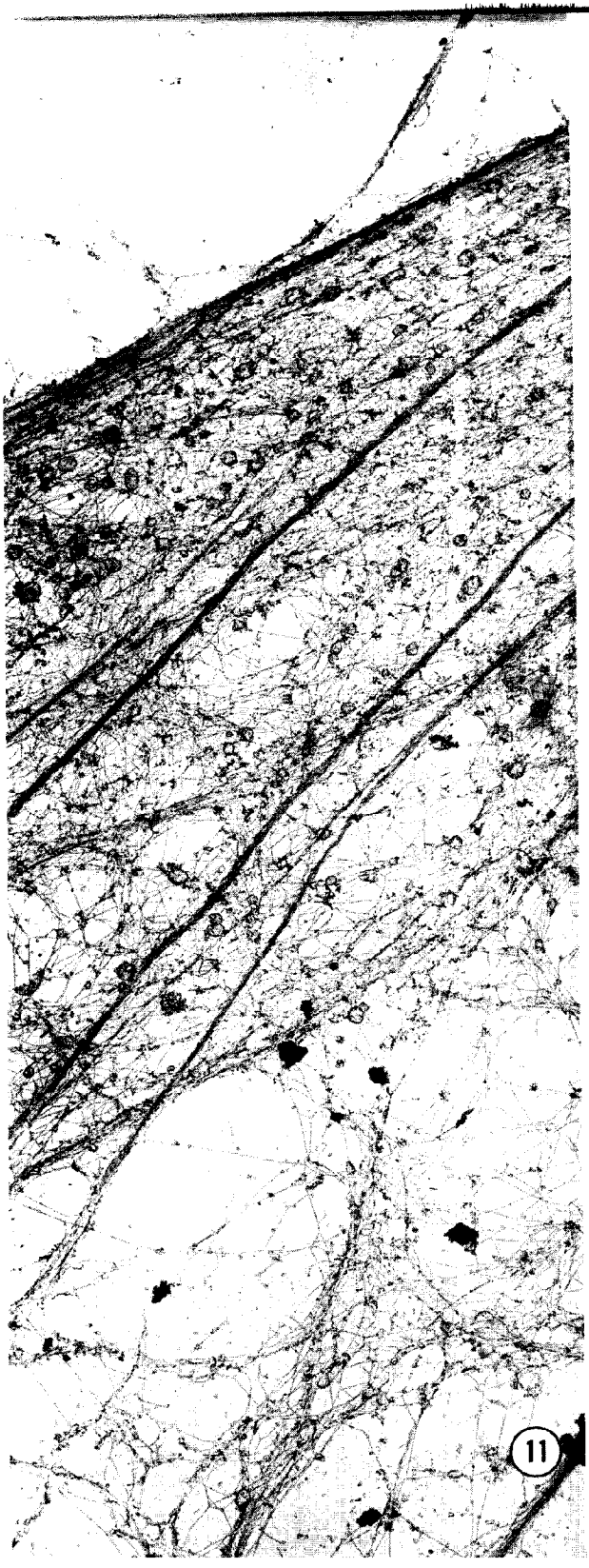
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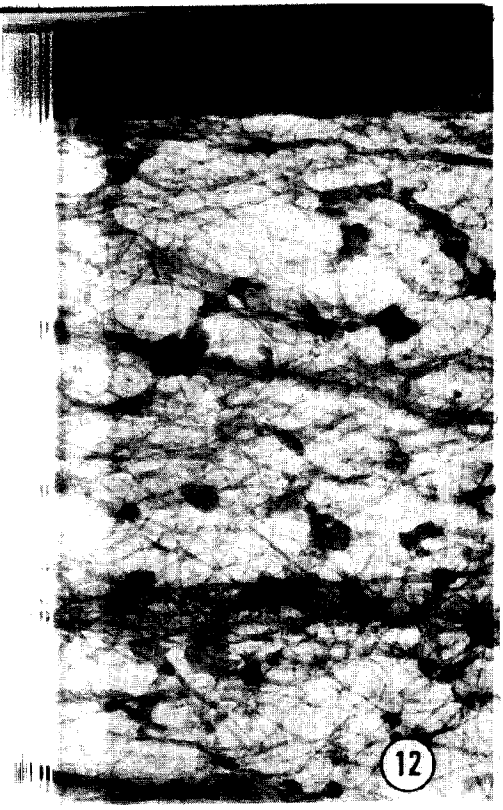
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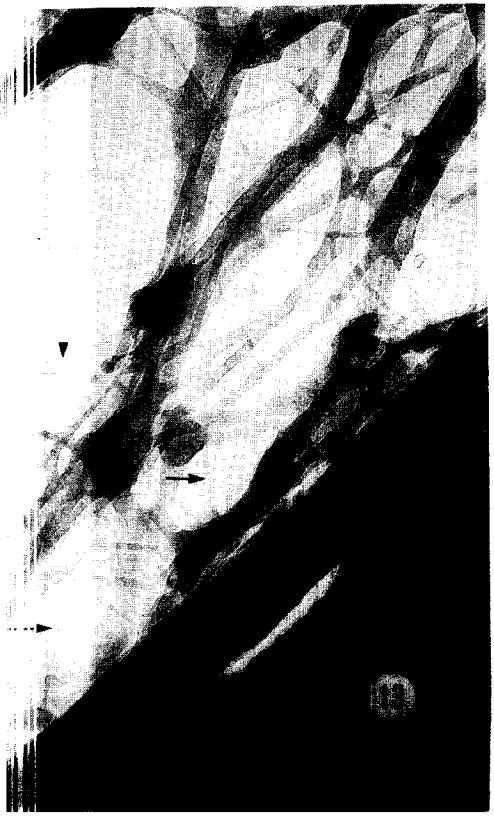

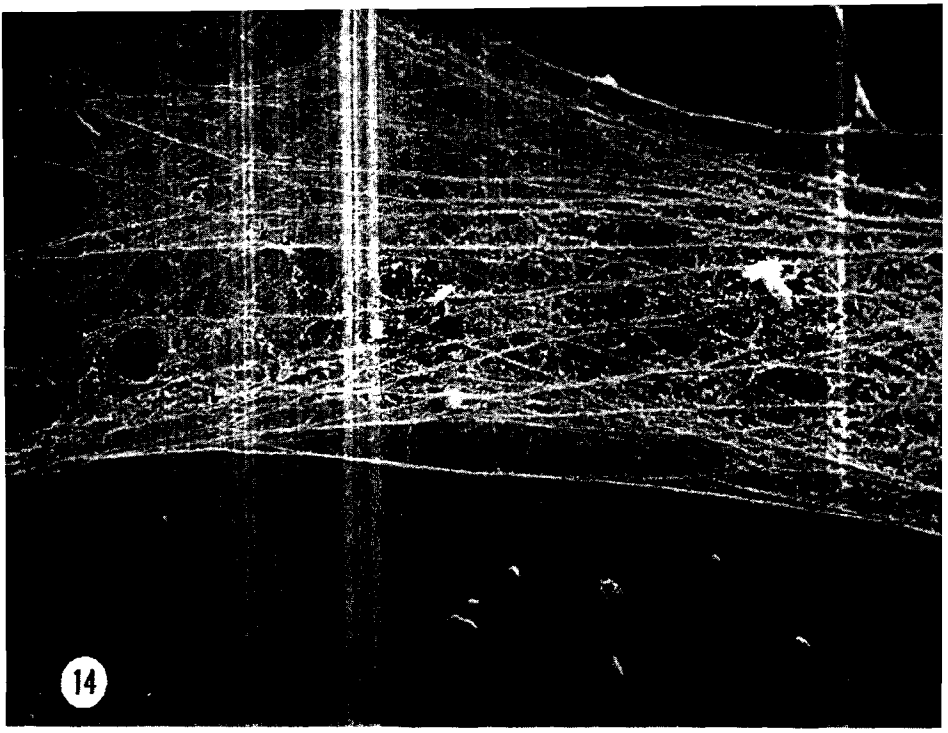
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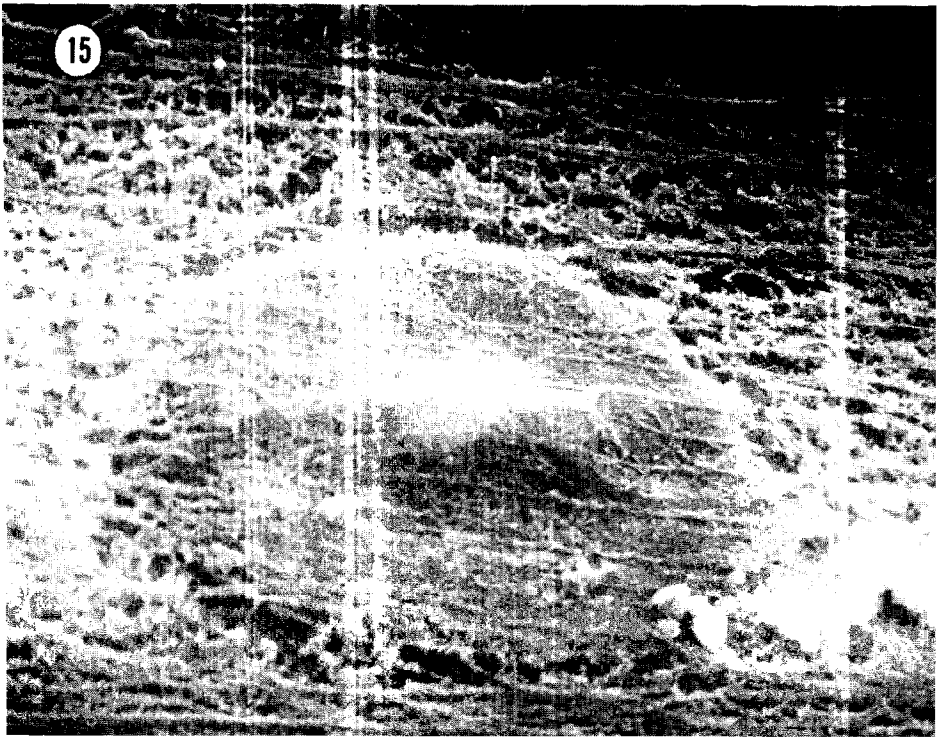
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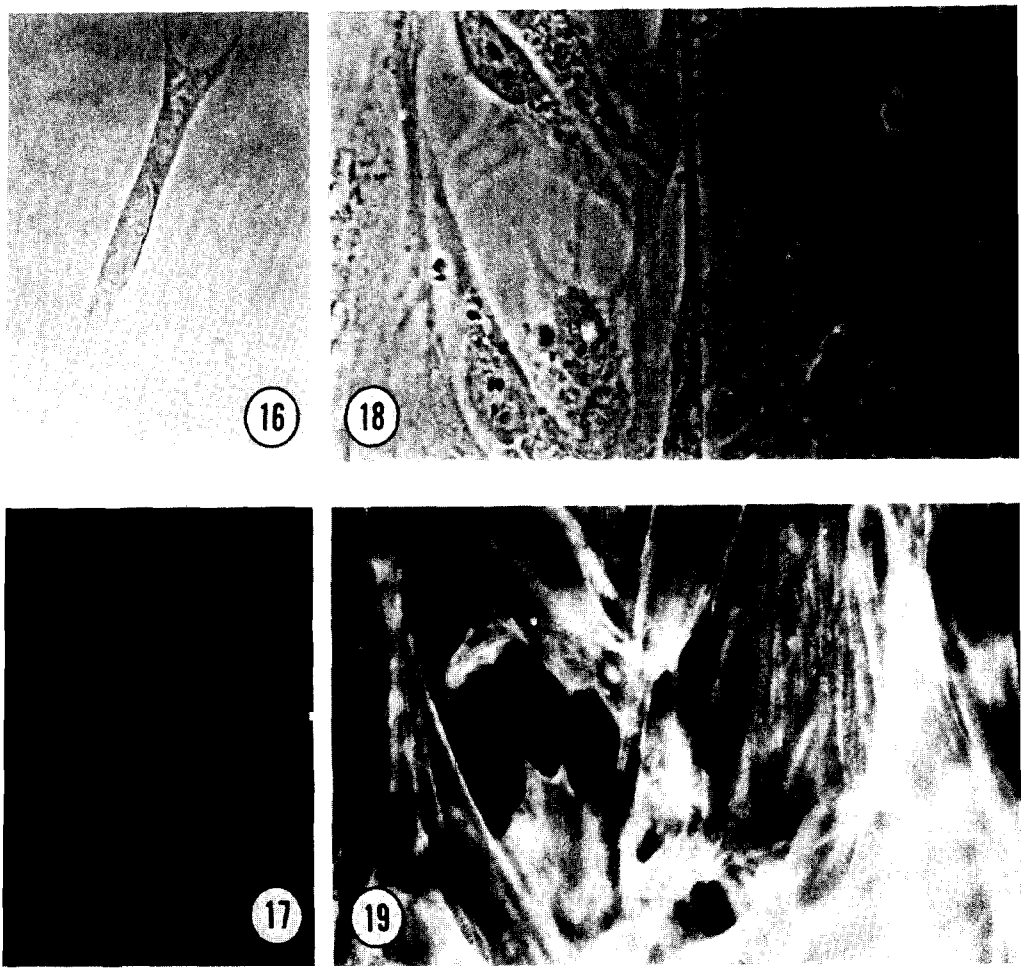

Fig. 16. Phase contrast micrograph of a presumptive chick myoblast. × 850.

Fig. 17. The same cell following incubation with human antiplatelet myosin conjugated to rhodamine. The myoblast has reacted positively, but weakly along the lateral edges of the cell. × 850.

Fig. 18. Phase-contrast micrograph of human myoblasts. × 850.

Fig. 19. The same cells after treatment with human antiplatelet myosin conjugated to rhodamine. These cells have reacted strongly and bright fluorescing structures were present along the lateral edges of these cells. × 850.

Fig. 14. Scanning micrograph of human myoblast showing the rich and complex system of filament bundles remaining after extraction with Triton X-100. × 3200.

Fig. 15. Scanning microscopy was very useful in determining the filamentous structures overlying the nucleus. In this extracted human myoblast, the nuclear region is overlaid with a reticulum of fine filaments and by many filament bundles. × 3850.

scopy affirmed the observations made with transmission electron microscopy (although at a lower resolution), and at the same time revealed more of the three-dimensional aspects of the filamentous organization.

Fig. 14 is a scanning electron micrograph of the complex organization of bundles of filaments in a detergent treated human myoblast. The thick accumulation of filamentous structures comprising the lateral edge cables are obvious, but the dense reticulum of microfilaments and its relationship with the filamentous bundles could not be resolved.

Detailed study of the nuclear region and the relations of the surrounding framework of filaments could not be elucidated by transmission electron microscopy because of the impenetrability of this area by the electron beam. Scanning preparations, however, were useful and revealed a reticulated organization of microfilaments overlying, and filament bundles traversing, the nucleus.

Antibodies

The characteristics of the antibody (a gift of K. Fujiwara) and the direct immunofluorescent technique have been presented elsewhere (Fujiwara and Pollard, 1976). When chick presumptive myoblasts were treated with human antiplatelet myosin conjugated to rhodamine the cells reacted positively but weakly due to the species specificity of this antibody. The most prominently reactive areas occurred at the peripheral regions of the cell (Figs. 16, 17). Human myoblasts, however, bound the antibody more strongly and exhibited many brightly fluorescing cables within the cells (Figs. 18, 19). As with chick myoblasts, the peripheral regions of human myoblasts reacted heavily with the antibody.

Discussion

The present study combines transmission and scanning electron microscopy of whole mounts of cultured chick and human myoblasts extracted with Triton X-100 to investigate the composition and organization of the intracellular filament bundles known previously as 'stress fibers'. These bundles formed a complex organization, frequently branching and anastomosing with each other. The bundles were continuous with the filamentous reticulum indicating that the whole filamentous organization of myoblasts was integrated. Therefore, any motility in one part of the filamentous cellular organization must be translocated to other parts of the cell. Such an integrated system allows functional or morphological changes to be recognized and acknowledged by all regions of the cell. Thus these filament bundles, while undoubtedly serving structural and physiological functions, must also serve as a communicative network throughout the cell, transferring stresses from one part to distant areas.

Further observations indicated that during the initial spreading and attachment of chick and human myoblasts the intracellular bundles of fibers varied greatly in their morphology and spatial arrangements. The most obvious structures were dense, tightly packed aggregations of filaments, which would have corresponded to 'stress fibers' seen by phase contrast microscopy. The diameters of these bundles varied considerably, from 30 nm up to 350 nm in presumptive chick myoblasts and three-dimensional imaging indicated that these structures mainly were present along the substratum of both types of myoblasts. These bundles of filaments traversed the remnant cell body, often for long distances and occasionally entered cellular processes that are extended by myoblasts during their differentiation. Apart from these distinct bundles of filaments there also occurred other smaller bundles composed of less tightly packed aggregations of filaments. These bundles of filaments were numerous and formed a seemingly unorganized pattern within the filamentous reticulum of human and chick myoblasts. Also many of these small bundles often became disorganized with the constituent filaments anastomosing with the filamentous reticulum. Furthermore, scanning electron microscopy of extracted human myoblasts also demonstrated that bundles of filaments traverse the nucleus. Thus apart from any direct morphological interrelationship that may occur between the nucleus and filament bundles, the nucleus itself is enclosed by a cage of filament bundles.

The most striking cable of filaments occurred along the lateral edges of both chick and human myoblasts. These dense lateral edge cables appeared structurally distinct from the other intracellular fibers present in

chick and human myoblasts. They were thicker and more tightly packed; in fact, these cables were so dense that the electron beam could not penetrate these structures, except for a fortuitous area in a human myoblast where it could be seen that these dense lateral edge cables were in part composed of large structures 40–50 nm in diameter. The identity of these structures is uncertain at the moment. Since, however, in both types of myoblasts the most intense labelling of fluorescent antibody to myosin occurred along the cellular margin, myosin may be associated with other filamentous proteins to form a major constituent of the lateral edge cables. The lateral edge cables were most obvious along those edges of the cell presumably not undergoing surface activity. This could indicate that these structures possess a stable composition and represent a permanent filamentous organization necessary to the further differentiation of myoblasts.

Microtubules were not apparent in whole mount specimens, unless these specimens were processed for thin-section microscopy whereupon microtubules were preserved and readily observed. These thin sections demonstrated that although microtubules often appeared oriented randomly they were also organized into bundles often associated with bundles of microfilaments. Microtubules were also seen to course along the peripheral margins of the extracted myoblasts and thus could possibly be components of the lateral edge cables. Microtubules, either singly or in bundles were also seen to be clearly associated with the nuclei of extracted myoblasts.

Following the attachment of chick myoblasts to the substratum further differentiation involves the elongation of the cells. During this period of elongation the bundles of filaments become dramatically rearranged and now course longitudinally to the axis of the cell body, the small short bundles are not so prominent. Eventually the myoblasts fuse to form myotubes and Triton X-100 extraction of cells which are possibly in the process of fusing indicate that the bundles of filaments may play a role in establishing physical continuity between these cells. Bundles of filaments of these cells enter the filamentous reticulum of adjacent cells and appear to become confluent with other filaments present in these cells. This process could be an important factor in not only establishing contact between fusing cells but also in the actual fusing process of these cells.

A multiplicity of function has been suggested for filament bundles in various types of cultured cells (Goldman *et al.*, 1976) which could suggest a correlated diversity in structure. It had not previously been established whether 'stress fibers' are functionally homogeneous or heterogeneous, or even whether their function can vary from one cell line to another. The present study demonstrates a spatial and structural heterogeneity among filament bundles in both chick and human myoblasts; long, tightly packed bundles of filaments present in the ventral and dorsal regions of the extracted cells (presumably corresponding to 'stress fibers'), shorter loosely aggregated bundles present within the filamentous reticulum, and large dense structures comprising the lateral edge cables. Furthermore, a role in the process of fusion could be ascribed to filamentous bundles since they appear to establish physical continuity between fusing cells. It is possible that these filament bundles may reflect functions as varied as their composition. It may further indicate that any of the filament bundles, when appropriately primed, may become either supportive or contractile elements, or both, in a dynamic process of continuous rearrangement or restructuring.

Acknowledgement

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