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Part 4

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PART IV

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The editor gratefully thanks the authors and reviewers (see p. vii-viii) for their contributions, invites your comments on ways to improve this procedure and seeks qualified volunteers to assist with reviewing papers in the future.

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PINOCYTOTIC VESICLES AND MICROFILAMENTS OBSERVED BY SCANNING ELECTRON MICROSCOPY.

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Abstract

Pinocytotic vesicles and microfilaments were clearly demonstrated by high resolution scanning electron microscopy.

Mesothelial cells covering the newt liver were excised and treated with a hypotonic phosphate buffer solution (150 mOsm) for 1-2 minutes. After fixation with 2.5% glutaraldehyde, some specimens were placed in 20% glycerin solution for 24 hours. After being postfixed with 1% osmic solution, they were treated with 2% tannic acid and 1% osmic solution for conductive staining. After dehydration in a graded series of ethanol, the deeper layers of the mesothelium were exposed by scratching or rubbing the specimen in absolute ethanol.

When some part of the surface cell membrane was turned over, many pinocytotic vesicles could be successfully seen attached to the cytoplasmic side of the membrane. The vesicles were also observed on the basal cell membrane. In the deeper layer of the cell exposed by scratching or rubbing the specimen, intracellular fibers presumably microfilaments were spread in the whole cell body forming "cytoskeleton".

KEY WORDS: Pinocytotic vesicle, Microfilament, Peritoneal mesothelium, Hypotonic treatment, Glycerin treatment.

Introduction

Peritoneal mesothelial cells are known to have absorptive activities on their free surface. The mechanism of absorption has been studied by transmission electron microscopy (TEM) and pinocytotic vesicles have been shown to be concerned with this mechanism (1,2).

Recently, intracellular fibers have received increased attention by cytologists. Microfilament systems and their relationship have been discussed with cellular motility (3,4). These intracellular fibers are referred to as the "cytoskeleton" consisting of microfilaments, microtubules and intermediate fibers. Scanning electron microscopic (SEM) studies of them have been carried out only on cultured cells using some non-ionic detergents like Triton X (5). In this method, the detergent removes part or all of the plasma membrane, while most of the cytoskeletal elements are retained. Since we cannot observe the fibers and membrane at the same time, interrelations between the two elements cannot be clearly studied. Recently the author has devised a preparation method using glycerin solution to remove some part of a cell membrane for observing intracellular fibers and pinocytotic vesicles (6). However, this technique may not be always successful and a revised method has been tested. The purpose of this paper is to describe the devised method using hypotonic buffered solution which can demonstrate pinocytotic vesicles and intracellular fibers consistently.

Materials and Methods

The animal used in this study was the adult Japanese newt (*Triturus pyrrogaster*) weighing about 5 grams. The observations presented here were obtained from peritoneal mesothelium lining the liver. The liver was freshly removed and excised in 3 x 3 mm square. Before fixation, the specimens were immersed in hypotonic 0.06 M phosphate buffer solution (150 mOsm), pH 7.2 for 1-2 minutes. Then, they were fixed by immersion for 2-3 hours at room temperature with 2.5% glutaraldehyde in the same buffer. Following a rinse in the same buffer for 20 minutes, some samples were placed in 20% glycerin aqueous solution for 24 hours at room temperature (6). They were rinsed again with the same buffer and postfixed with 1%

osmic solution in the same buffer for 1 hour at room temperature and conductive-stained as follows. They were rinsed in the same buffer for 30 minutes or more, and immersed in 2% tannic acid aqueous solution for 12 hours at room temperature. After they were washed in the same buffer, they were stained with 1% osmic solution in the same buffer for 30 minutes at room temperature. After rinsing in the same buffer, the specimens were dehydrated in a graded series of ethanol. Then, the deeper layers of the mesothelium were exposed in absolute ethanol either by scratching the mesothelial surface using a pin or rubbing the surface of the specimen. When rubbing the surface, two specimens were held with two forceps and the mesothelial surfaces were rubbed against each other gently in absolute ethanol. Then they were critical point dried using liquid CO_2 (7) and coated with platinum in a sputter coater with a rotating stage (VX-10R, EIKO Engineering Co., Ltd., Japan). The samples were examined in a field emission scanning electron microscope (HFS-2ST, Hitachi Co., Ltd., Japan), operated at 25 kV.

Results

The use of hypotonic buffer and glycerin solution removes a part of the cell membrane, thus revealing intracellular structures underlying the cell surface. Though the use of glycerin solution is not necessary to reveal intracellular structures, it removes the cell membrane more efficiently. Moreover, the deeper layer of the cell is observed by scratching or rubbing the specimens artificially in absolute ethanol (Fig. 1, 2). Figure 1, from an artificially scratched mesothelial cell shows a surface cell membrane (SM), its reverse side (arrow), the layer just under the surface cell membrane (SL) and exposed deeper layer of the cell (DL). Figure 2 also shows three layers of an artificially fractured cell by rubbing the surface of the specimen: surface cell membrane (SM), intracellular fibers (IF) and basal cell membrane (BM).

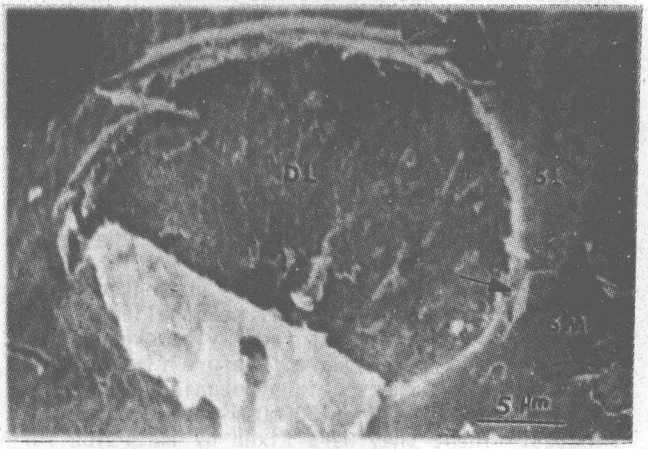

Fig. 1 Mesothelial cell surface and intracellular structures exposed by hypotonic lysis and scratching the specimen using a pin, showing a surface cell membrane (SM), its reverse side (arrow), the layer just under the surface cell membrane (SL) and the exposed deeper layer of the cell (DL).

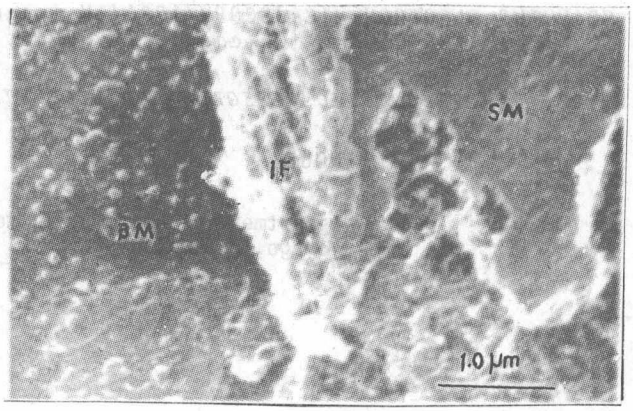

Fig. 2 Mesothelial cell fractured artificially in absolute ethanol by rubbing the mesothelial surface, showing a surface cell membrane (SM), intracellular filamets (IF) and a basal cell membrane (BM).

Fig. 3 High magnification of the mesothelial cell surface. Many surface pits (arrowhead) and shallow depressions (arrow) are seen on it.

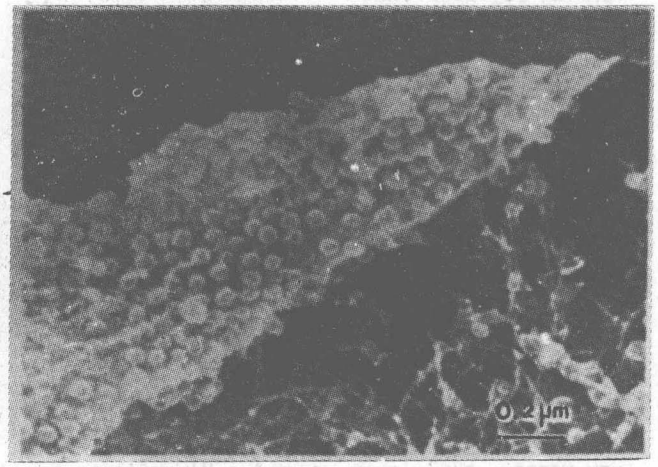

Fig. 4 A part of the surface cell membrane is found to be turned over by hypotonic lysis, and the cytoplasmic side of the membrane is seen. Many pinocytotic vesicles are attached. Just under the membrane, microfilament network is visible.

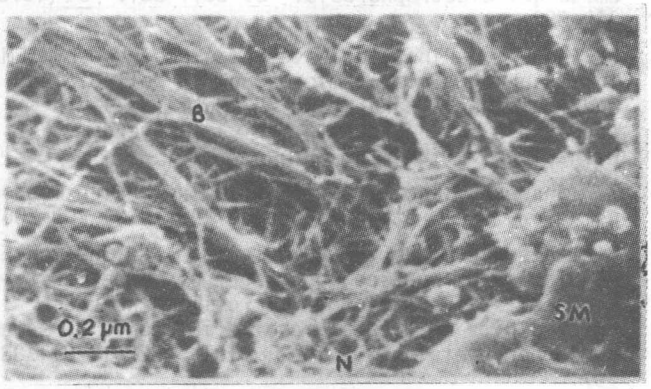

Fig. 5 Microfilament network (N) and bundles (B) under a surface cell membrane (SM). Microfilament network is seen just under the cell membrane and microfilaments form parallel bundles under the network.

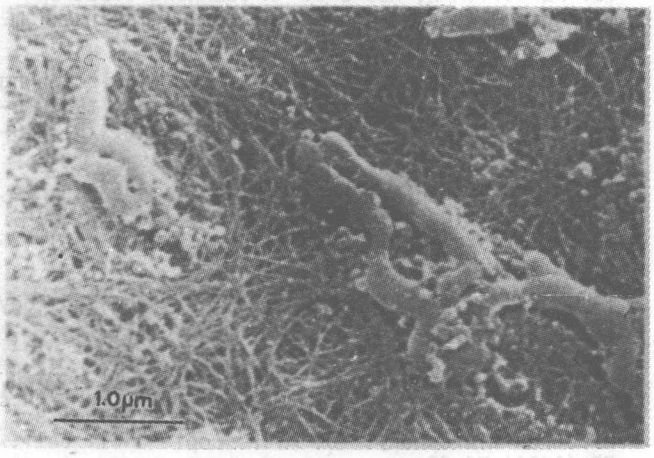

Fig. 6 High magnification of the deeper layer of the cell (Fig. 1). Mitochondria are observed in microfilaments which tend to form bundles around mitochondria.

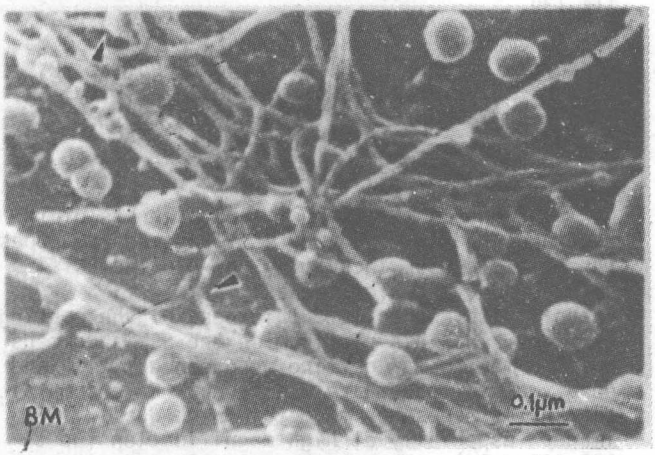

Fig. 7 The cytoplasmic side of a basal cell membrane (BM). Microfilaments and pinocytotic vesicles are observed. The ends of the filaments are attached to the membrane and pinocytotic vesicles (arrowheads).

The mesothelial surface is populated with many pits and shallow depressions (Fig. 3). All these pits are nearly of the same size (15-20 nm in diameter) and are presumed to be orifices of pinocytotic vesicles. Shallow depressions of about 100 nm diameter are also occasionally seen.

When some part of the surface cell membrane is found to be turned over because of hypotonic lysis, the cytoplasmic side of the membrane is observed. Lots of vesicular structures of pinocytotic vesicles (50-80 nm in diameter) are attached (Fig. 4). Under a surface cell membrane, many filamentous structures (15-20 nm in width), presumably interconnected microfilaments are clearly seen (Fig. 4). Microfilaments form a filamentous network just under the surface cell membrane, while they tend to form parallel bundles under the filamentous network (Fig. 5).

In the deeper layer of the cell exposed by scratching (Fig. 1; DL), mitochondria are observed in the filamentous structures which may be microfilaments (Fig. 6). There, these filaments tend to form bundles around mitochondria.

In the basal portion of the cell, pinocytotic vesicles also attach to the cytoplasmic side of the basal cell membrane (Fig. 7). Microfilaments are also visible forming bundles of interconnecting network, some of them are attached to pinocytotic vesicles and to the inside of the basal cell membrane (Fig. 7). The inside of the basal cell membrane is not smooth, but has fine granular substances on its surface.

Discussion

Pinocytotic vesicles have been studied in detail by TEM (1,2,8,9) and freeze replica technique (9). However, their SEM studies have been limited to the orifices of the vesicles (8,10). Singer (9) showed that pinocytotic vescicles on the cell surface of the fibroblasts were frequently arranged in ordered linear or fusiform aggregates, which resulted from interactions between microfilament bundles and plasmalemma. In the present study, pinocytotic vesicles are distributed randomly, and microfilament bundles cannot be discerned just under the plasma membrane. Madison et al. (8) reported the depression termed vesiculated pits on which pinocytotic vesicle openings were visible and that these depressions were restricted to areas of the cell surface containing microvilli. On the shallow depressions observed in our study, no openings of pinocytotic vesicles are visible and no microvilli exist on the cell surface. Therefore, these shallow depressions may not be vesiculated pits.

The present study clearly demonstrates intracellular fibers by SEM using hypotonic solution and glycerin. Previous SEM observations of cytoskeleton have been mainly carried out on cultured cells extracted with some detergents. But, relations between fibers and membrane have been obscure because of a membranous components being dissolved. Ip (11) identified three types of cytoskeletal structures (actin filaments, intermediate filaments and microtubules) and immature elements of the sarcoplasmic reticulum within cryofractured muscle cells cultured from embryonic

chick by SEM. Heuser (12) showed actin, microtubules and intermediate filaments by rapidly frozen, freeze-dried techniques. In the present study, intracellular fibers seem to be microfilaments, and other elements of cytoskeleton are not identified. According to my TEM study of the newt mesothelial cells (not shown here), most of the intracellular fibers have proved to be microfilaments. Since Buckley(13) began the observation of whole cultured cells by TEM with critical point drying method, the structures and constitutions of cytoskeleton have been clarified stereographically (14, 15). Goldman et al. (16) showed microfilaments in cultured cells were often found to be arranged in two distinct morphological modes; fibers composed of closely packed parallel microfilaments, and network of non-parallel microfilaments. The present study demonstrates microfilament network and bundles in various layers of the cell. Under the plasma membrane, microfilaments form interconnecting network structure, while in the deeper layer of the cell they tend to form bundles. Wolosewick et al. (15) proposed the model of intracellular fibers. According to them, the cytoplasmic ground substance of cultured cells consists of slender strands that form an irregular three dimensional lattice (microtrabecular lattice), which is continuous with cortex of the membrane and with stress fibers and microtubules. The filamentous network under the plasma membrane obtained in the present study may correspond to the "microtrabecular lattice". On the inside of the basal cell membrane, some fine granular strucutres are visible. Then this layer with the fine granular substances may be the "cortex" described by them. They (15) also showed a part of the microtrabecular lattice was continuous with the plasma membrane. In the present study, microfilaments are shown to be connected to the "cortex" of the membrane and to pinocytotic vesicles.

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Discussion with Reviewers

W. Ip: What is the approximate thickness of the sputtered platinum and why is it necessary? Does the multiple OsO_4 incubation not provide sufficient conductance for the samples?

Author: The approximate thickness of the coating is 5 nm. The thin platinum on the SEM specimens has two merits for getting good conductivity and good yield of secondary electrons. So the metal coating is necessary to obtain high quality SEM images, especially at high magnification (up to 100,000 fold). Though the multiple OsO_4 incubation provides better conductivity to the specimen, intracellular structures might be destroyed by the multiple incubation. Therefore, the specimens should be conductive stained one or two times at the maximum.

R. G. Kessel: Can you indicate more precisely the effect of the preliminary immersion in hypotonic phosphate buffer?
Author: The immersion in hypotonic buffer before fixation would cause cell lysis and probably egress of soluble cytoplasm. When the matrix substance is not removed before fixation, intracellular structures are not exposed. If not, they are concealed by the fixed matrix substance.

W. Ip: Is the use of glycerol really necessary, since incubation in hypotonic buffer alone should be sufficient to cause cell lysis and egress of soluble cytoplasm?
R. G. Kessel: Can you indicate more precisely the effect of immersion in 20% glycerin solution without the prior buffer solution?
Author: The use of glycerin solution is not necessary to reveal intracellular fibers, but it removes the cell membrane more efficiently. Though a short time immersion in the glycerin solution without the prior hypotonic buffer treatment can not remove a surface cell membrane, it is possible to remove a part of the membrane by a long time glycerin treatment.

Editor: Reviewers of the paper by Panessa and Gennaro (Scanning Electron Microsc. 1973: 395-402) indicated that use of glycerol can cause extensive contamination in the SEM column. What has been your experience?
Author: I have not encountered such problem yet.

J. A. Trotter: Could the membrane deformation you see (caveolae, pits, vesicles) be an artifact of the glycerin or glutaraldehyde treatment?
Author: No, Many TEM studies have shown that peritoneal cells have active absorptive activities in the form of pinocytosis.

J. A. Trotter: "Pinocytotic vesicles" may all be caveolae.
Author: Strictly speaking, "Pinocytotic vesicles" are caveolae, but the term "pinocytotic vesicles" is widely used when discussing peritoneal cells.

R. G. Kessel: How does the size of the pits (15-20 nm) and vesicles (50-80 nm) as measured in your SEM preparations compare to those observed in TEM studies?
Author: According to my TEM studies of mesothelial cells, the size of the pits is 13-20 nm in diameter, while that of vesicles is 60-90 nm in diameter.

J. A. Trotter: Do you see any coated pits with this technique?
Author: Yes, I have observed the coated vesicle which has a spherical polygonal "basket work" by SEM in the different materials using the same method. I could not observe coated vesicles with "polygonal basket work" in the present study.

I. I. Singer: Fig. 3 depicts the external openings of pinocytotic vesicles which appear lined up in rows, especially at the left edge of the micrograph, and to the right of the arrowhead. Indications of a linear distribution are also seen in Fig. 4. I do not see why the author states that the vesicles are randomly distributed.

Author: As you have pointed out, some of the pits and vesicles appear lined up in rows. But, I think that they are distributed more randomly than those which you have shown in your paper (9).

I. I. Singer: In Fig. 6, the "mitochondria" could be endoplasmic reticulum.
Author: I think the long, meandering and branching structures in Fig. 6 are mitochondria. Because, endoplasmic reticulum in the newt peritoneal cell shows star-like flattened shape, so far as I have observed (not shown in this paper).

J. A. Trotter: When examining the basal cell membrane, do you see regions with an appropriate morphology to be "adhesion plaques" where the cell would be attached to deeper structures? What is the nature of mesothelial attachment in this system?
Author: I have observed disk-like structures (300 -450 nm in diameter) on the basal cell membrane. Thus far, it is obscure whether this structure may correspond to adhesion plaques. Precise studies are now being performed.

J. A. Trotter: Do you ever see cell-cell junction using these techniques?
Author: No, not yet.

I. I. Singer: The filamentous structures shown in this work are described as microfilaments, implying that they are actin containing. At the level of resolution employed, and especially with platinum coating, it would be difficult to distinguish microfilaments from intermediate filaments on morphological criteria alone.
J. A. Trotter: I don't see how you justify your statement that all the filaments you see in this study are "microfilaments", i.e., actin filaments.
R. G. Kessel: Are filaments observed in similar locations in the mesothelial cells in thin sections with the TEM? What is their diameter?
Author: As you pointed out, it is difficult to identify microfilaments on the basis of morphological criteria alone. However, according to my TEM studies of peritoneal cells, the intracellular fibers observed in similar location in the present study were microfilaments (5-6nm in diameter). My TEM observation showed intermediate filaments and microtubules were distributed around the nucleus. Judging from the location of the filaments, I think intracellular fibers observed in the present study are microfilaments.

J. A. Trotter: Apparently when you "scratch" the cell, it separates into layers. Does this separation occur in a similar fashion in all cells? In other words, does it allow you to define layered compartments of the cell, the interfaces between which are the weak places where separation occur?
Author: The separation occured almost in a similar fashion. Sometimes the separation occured on the top of the nucleus or on the top of the surface of the basal lamina. So, I think the layered compartments of the cell would be defined in the precise study.

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For Immediate Release

THE SCIENCE OF BIOLOGICAL SPECIMEN PREPARATION FOR MICROSCOPY AND MICROANALYSIS will be the subject of a **special conference** from **April 23 to 28, 1983** at the Sugar Loaf mountain resort located near **Traverse City** (in the scenic area of **northwestern Michigan**, about 240 miles from Detroit).

Prof. Jean-Paul Revel (Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125, phone 213-356-4986, as the general chairman), **Dr. Tudor Barnard**, Oslo University, Norway (phone 02-456191), **Dr. Geoffrey H. Haggis**, Agriculture Canada, Ottawa (phone 613-995-3700 X219), and **Prof. Takuro Murakami**, Okayama University Medical School, Japan (phone 0862-237151) are the organizers of this program.

Interested contributors should immediately contact one of the organizers; for contributed papers a 200 word abstract is required by January 15, 1983. Attendance at the conference will be by application (form available on request) or invitation only.

The program will deal with our present **understanding and rationale (no recipes) for manipulations** carried out during biological specimen preparation for microscopy **(including light optical, SEM, TEM, STEM, etc.)** and microanalysis **(X-Ray, LAMMA, etc.)**. Among topics to be covered by the experts from United States, Canada and abroad are: *chemical and cryo-fixation, dehydration (including freeze drying and critical point drying), embedding, sectioning, cryo-sectioning, freeze cleaving, preparation of whole mounts, coating, general staining techniques, preparation of macromolecules, etc.*

The formal sessions of the conference will be in the mornings and nights. Each session will consist of two to four presentations with ample time scheduled for discussion and participation by the audience. In the afternoons time will be provided for organized informal discussions, meetings of special interest groups, and presentations of posters.

The proceedings of the conference will be published, additional details will be included in the next announcement.

Traverse City has direct flight service from Detroit and Chicago. The conference center has many indoor and outdoor activities, as well as many sightseeing possibilities in the nearby areas.

This is the second program of the **Pfefferkorn Conferences Series and is sponsored by SEM Inc.** This conference follows the Scanning Electron Microscopy/1983 meeting scheduled from April 17-22, 1983 at the Hyatt Regency Hotel in Dearborn, Michigan (just outside of Detroit).

For additional information about this conference or SEM/1983 contact Om Johari at the address given above.

SCANNING ELECTRON MICROSCOPY/1981/IV (pages 7-15).
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THREE-DIMENSIONAL OBSERVATION ON MUSCULAR TISSUES

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Abstract

Striated muscle cells of frog sartorius and smooth muscle cells of guinea pig taenia coli were observed three-dimensionally by field emission type scanning electron microscope (FE-SEM) and freeze-fracture deep-etching replica (FFDE) method.

By tearing at different preparative stages, various cellular structures appeared on SEM : the outer surface of cells, the inner surface of plasmalemma, and the interior of cells. On the inner surface of the plasmalemma of striated muscle, many caveolae, membranous structures and fine filaments were observed. Inside the cells myofibrils showed the characteristic banding pattern and on the thick filaments, a periodicity of 40 nm was observed. Along the myofibril, T-tubules and sarcoplasmic reticulum were seen with their regional differentiations.

In smooth muscle cells, on the inner surface of the plasmalemma there were many caveolae, bundles of filaments and endoplasmic reticulum. The central part of smooth muscle cells were occupied by their nucleus, many microtubules, mitochondria and rough surfaced endoplasmic reticulum.

By the FFDE method, observations on finer details were added to the SEM studies, including lateral projections of myosin filaments in striated muscles and fine filaments connecting thin myofilaments in smooth muscle cells. In the terminal cistern of the sarcoplasmic reticulum, networks composed of granular materials were observed.

KEY WORDS: Scanning electron microsopy, Freeze fracture deep etching replica method, Sartorius muscle, Taenia coli, Striated muscle, Smooth muscle, Caveolae, Sarcoplasmic reticulum, Myofilament, T-tubule, Microtubule

Introduction

Muscular tissues are special for their contractile function and show a high level of organization especially in striated muscle. Observation of these beautifully organized structures three-dimensionally is an attractive objective for investigation. In the past, the use of conventional transmission electron microscopy made it difficult to observe the exact ultrastructural three-dimensional relationship of organells due to the compact packing of cell organells. It has only been possible to observe the overlapping of various structures by this technique. To overcome this, high resolution three-dimensional techniques or observation of surface structure is needed. In fact, recent progress in scanning electron microscopy (SEM) partly enabled us to perform such observations (24). Furthermore freeze-fracture deep-etching replica method provided additional means for such observation with much higher resolution (12).

SEM studies of past 10 years

More than ten years ago, the early scanning electron microscopists started SEM study on muscular tissues (2,30). They observed the outer aspects of muscle fibers after solubilizing extracellular collagen with collagenase. Haggis (9) tried to observe the intracellular structures of muscle fibers by cryofracture. Since then several methods have been used to observe the interior of muscle cells; cryofracture (1,15,25,26,27), deparaffinized specimens from paraffin embedded sections (18,22), knife cut specimens (3,7,8), and etched specimens from Epon sections (19). By these methods, in cardiac muscles, these authors observed rows of mitochondria (3,15,18,22,26), myofibrils, and thickened structures at Z-disk levels (1,3,15,18,22,26). The latter structures were sometimes connected to the plasmalemma (26,27) and thought to correspond to T-tubules. However their exact contour was not clarified. Some authors (18,22) observed the network of sarcoplasmic reticulum, which showed as rough networks around the myofibrils. The fractured interior and outer aspect of nucleus was also observed (26,27). The outer surface of nucleus was covered with tubular and granular structures. The nucleoplasm appeared as fine networks and the nucleolus was seen as dense structures. In skeletal muscles. the thickenings of myofibrils at

Z-disk levels were also observed (6,7). In mouse skeletal muscle (7) structures which possibly should have been interpreted as mitochondria at the A-I junctions were seen in some SEM micrographs. Although the T-tubules and sarcoplasmic reticulum were mentioned their fine structures were hardly recognizable in SEM micrographs of paraplast-embedded material. On smooth muscle cells, there have been no SEM studies except on the contour of isolated cells by Fay and Delise (5).

Three-dimensional studies other than SEM

Several methods for three-dimensional studies on cells and tissues have been in use. Reconstruction from serial sections with transmission electron microscopy and stereo-paired photographs from thick sections observed by high voltage electron microscope are notable ones. However, they cannot give the direct three-dimensional images. Freeze-fracture methods with glycerol also provided the three-dimensional information which was limited to the membranes.

Recently, a method of deep-etching without cryoprotectant was used by Heuser and Salpeter (12). They showed three-dimensional photographs with very high resolution. In their method, only a 20 μm thickness of the specimen surface was free from ice crystals and could be used for study, although the cells were cryo-fixed in living state. This causes some limitations for the application of the techniques. We tried to find a method to make ice crystal free region much wider by using sublimable cryoprotectant although we used the chemical pre-fixation.

The present paper describes observations made on the three-dimensional nature of muscular tissues using field emission SEM (FE-SEM) and freeze-fracture deep etching replica (FFDE) method now in progress.

Methods

Methods for high resolution study by FE-SEM

With the appearance of FE-gun or LaB_6 gun, the resolving power of SEM has been much improved. Accordingly, the preparative techniques of specimens must be devised for the higher resolution. The sputter coating method extremely reduced the island formation produced by coating metals. In addition, the conductive staining by tannic acid (16) or thiocarbohydrazide (14) made it possible to minimize the thickness of metal coatings so that the fine details of structures are not covered and obscured by metal coatings. Since no studies have been reported on muscular tissues with high resolution SEM other than ours (23,24), the following sections deal with our observations on specimens prepared for high resolution SEM.

Methods for outer aspects of the cells The cells were fixed with 1% formaldehyde and 1.3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) by immersion at 4°C (frog sartorius muscle) or by perfusion and immersion at the same time at 37°C (guinea pig taenia coli). Both muscles were stored in fresh fixatives at 4°C for at least 12 hours. Then the tissues were torn along the cell long axis with two sharp forceps. By this procedure the tissues were torn preferentially at intercellular spaces. After splitting, the specimens were rinsed with 0.1 M cacodylate buffer, immersed in 2% tannic acid (buffered with 0.1 M cacodylate buffer and the pH was adjusted to 6.8 by NaOH) at 4°C for 6 hours. After washing with 0.1 M cacodylate buffer a minimum of 5 times with 15 min between changes, the tissues were post-fixed with 1 or 2% osminm tetroxide buffered with 0.1 M cacodylate buffer (pH 7.3) at 4°C for 6 hours. Then the tissues were dehydrated in graded concentrations of ethanol, substituted with iso-amylacetate and dried in a Hitachi HCP-1 critical point dryer. Dried tissues were coated as thin as possible with Au-Pd in an Eiko IB-3 sputter coater and observed in a Hitachi S-700 field-emission SEM operating at 15-20 kV. Although some of the specimens were treated with glycine-sodium glutamate-sucrose solution and with unbuffered solutions of tannic acid and osmium tetroxide according to the Murakami's method (17), no differences were seen in specimens treated in this way.

Inner aspects of the cells To reveal the interior of the cells several methods were tried. For example freeze-cracking method (29) or resin-cracking method (28). However the surface obtained by these methods was too flat for three-dimensional observation. We found that simple tearing after drying was very suitable for the aim in muscular tissues. The tissues were treated in the same manner as are outer aspect of the cells, except that tearing after pre-fixation was omitted and instead tearing was done after critical point drying. This enabled us to reveal the interior of the cells more successfully due to the tannic acid-osmium tetroxide treatment strengthening of the intercellular material.

Methods for FFDE

Fixed tissues were rinsed in 0.1 M cacodylate buffer for at least 15 min and immersed in 65% ethanol for 15 min. Then they were frozen in liquid nitrogen, fractured in an Eiko FD-2 freeze-fracture apparatus and then etched for 40 min at a setting temperature of -95°C. The etched specimens were rotary shadowed with platinum and carbon with shadowing angles of 60 degrees. Then the tissues were passed through distilled water and dissolved in household bleach. After the tissues were dissolved, the floating replicas were washed two times with distilled water and observed in a Hitachi HU-12A transmission electron microscope operating at 100 kV.

In this technique ethanol was used as a sublimable cryoprotectant. If the concentration of ethanol was higher than 65%, the tissues were too soft to be fractured. If the concentration was lower, ice crystals appeared in the central part of the specimen. When the fracture was made at liquid nitrogen temperature, the fractured plane was very smooth. When the temperature was slightly higher, the material showed some tendency to be split along the unit membrane just like the conventional freeze-fracture with glycerol. In our specimens the latter condition was used to reveal specific surfaces. Stereo-pair photographs (taken at tilting angle of 10 degrees) enabled us to make more exact three-dimensional observations.