Human Fibroblast Intracellular Network Prepared with Digitonin for Field Emission Scanning Electron Microscopy*

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Summary. Results obtained by extracting human fibroblast cells for the study of cytoskeletal structures are compared. Cells grown in culture were treated with digitonin in contrast to other methods of detergent preparation using Triton and Saponin. The three dimensional intracellular network which resulted from digitonin treatment was found to be similar in appearance to the structures observed by high voltage transmission electron microscopy of untreated cells described as a microtrabecular lattice by PORTER and TUCKER (1981). Our results, obtained by high resolution, field emission scanning electron microscopy indicate that the microtrabecular lattice may indeed be one conformation of a dynamic cytoskeleton.

Advances in the development and design of high resolution field emission scanning electron microscopes (SEM) have achieved instrumental resolution beyond 1 nm and are now approaching less than 0.5 nm. However, major problems in imaging at this level of instrument resolution remain in how to prepare a specimen to actually obtain similar specimen resolution. Our present specimen preparation techniques are formulated to preserve the structural integrity of proteins in an attempt to obtain as much information as possible before the effects of the electron beam, including charging, heating and placing in a vacuum combine to reduce the specimen to ashes. A most important side effect is beam-induced specimen motion which becomes increasingly more pronounced when observing biological samples at high magnifications. Beam irradiation may result in broken co-valent bonds and, to counter this effect, the specimen may be destroyed by actual mass loss. In order to prolong the survival time of the proteins under the electron beam, the specimen should be irradiated as little as possible. If only a fraction of the specimen is exposed per unit time and this beam spot is rastered over the specimen, induced motion can be minimized and a spot-scan image recorded (DOWNING, 1991).

While combining improvements in both SEM design and specimen preparation techniques, TANAKA, beginning in the early 1970's, used field emission high resolution scanning electron microscopy to take advantage of the spot-scan mode and special specimen preparation techniques. TANAKA (1972) pioneered specimen preparation techniques for the chemical removal of cytosol proteins with weak solutions of osmium tetroxide (PORTER and KALLMAN, 1953) to clearly display a three dimensional, stereo perspective of intracellular organelles. TANAKA, his co-workers and other investigators (LEA and HOLLENBERG, 1989) have, since that time, contributed much to the present knowledge and understanding of cellular microscopy. TANAKA demonstrated intracellular structures by high resolution scanning electron microscopy (TANAKA, 1981), further refined this method (TANAKA and MITSUSHIMA, 1984), applied freeze substitution specimen preparation for SEM (OSATAKE et al., 1985), optimized ion beam deposition (MITSUSHIMA et al., 1985), developed a new ultra high resolution scanning electron microscope (TANAKA et al., 1985), applied this SEM to biological samples (TANAKA et al., 1986, 1989) and observed ribosomes and macromolecules in situ (MITSUSHIMA et al., 1986).

A detrimental side effect due to the use of dilute solutions of osmium tetroxide for the removal of cyto-

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solic proteins was the complete loss of the cytoskeleton including microfilaments, intermediate filaments and microtubules (Maupin-Szamier and Pollard, 1978). Several methods have been tried to preserve the cytoskeleton, including mechanical fracturing, dry cleaving and removal of the cytoplasm by detergent extraction (for review see article by Bell et al., 1989). The effects of digitonin on hepatocyte cytoskeletal elements were reported by Fiskum et al. (1989).

We have used digitonin on a human fibroblast cell line grown in culture, in an attempt to expose the cytoskeletal components for SEM while maintaining the three dimensional relationships of the cytoskeletal components.

MATERIALS AND METHODS

Transmission electron microscopy
Fibroblasts were grown on formvar, carbon coated gold 3 mm grids, under sterile conditions. Once fibroblasts had attached to the surface of the formvar, the cells were taken through the fixation process. The cells were fixed in 2% glutaraldehyde for 1 h, followed by 1% osmium tetroxide. Dehydration was in a graded ethanol series and the grids were critical point dried using carbon dioxide as the transitional fluid (Lea and Ramjohn, 1980).

The fibroblasts were observed in a Hitachi H-7000 transmission electron microscope, at an accelerating voltage of 125 kV. The grids were carefully scanned and thin, podocytic processes of the cells containing few or no visible organelles were recorded as stereo pairs on sheet film.

High resolution scanning electron microscopy
Fibroblasts cultured from a human skin biopsy were grown on glass cover slips and maintained under sterile conditions.

Digitonin Extraction Medium (DEM) was made up by dissolving 0.5 mg/ml of digitonin (Digitonin #D-1407, Sigma Chemical Co, St. Louis, MO, USA) in buffer containing 0.25 M sucrose, 20 mM MOPS (3-[N-Morpholino] propanesulfonic acid) and 1 mM EDTA (Ethylenediaminetetraacetic acid).

Once the cells reached confluency, the medium was aspirated and the culture rinsed with 2 ml of phosphate buffered saline (PBS) and aspirated. 1 ml of DEM was then added to the fibroblasts attached to cover slips and left for 1 min. The DEM was then aspirated and the cells rinsed with 5 ml of PBS.

The cells were fixed for 30 min in 0.5% glutaraldehyde and 0.5% paraformaldehyde in M/15 phosphate buffer. The cells were washed, post fixed (30 min, in 1% osmium tetroxide), washed again and conductively stained (1% tannic acid in distilled water for 15 min), washed once more, followed by 15 min in 1% osmium tetroxide.

Fig. 1. 125 kV Transmission stereo pair electron micrograph of a pseudopod from a human fibroblast grown on a carbon coated, formvar gold grid. The process has several stress fibres (arrow) connecting it to the main body of the cell. The intracellular network forms a regular pattern in the cytoplasm and appears to be denser around the periphery. The process appears to be devoid of intracellular organelles except for three vesicular inclusions. ×2,700
The cells attached to cover slips were washed in the buffer, followed by dehydration through a graded then ethanol series and then critical point dried (LEA and RAMJOHN, 1980).

The cover slips were mounted on stubs with graphite suspension and sputter coated with a thin layer of gold. Some of the fibroblasts were dry fractured using sticky tape. The cells were viewed with a Hitachi S-4000 field emission scanning electron microscope (FESEM) and micrographs recorded as stereo pairs on Polaroid positive/negative film.

RESULTS

The fibroblasts grown on carbon coated formvar gold grids had extended characteristic processes along the carbon formvar surface. A typical process is shown in Figure 1. Despite this process being much thicker than a typical TEM section, it was possible, at 125 kV, for the electron beam to penetrate the cell's process and reveal an extensive intracellular network. The process itself appeared to be connected to the main cell body by many stress fibres. The density of meshwork elements was greater around the periphery of the process. Only three organelle-like structures were seen in this cell process, each containing small, spherical structures. The pattern formed by the network appeared to be quite uniform and symmetrical within the pseudopod.

When observed at a higher magnification in the TEM, the cytoskeleton formed a characteristic, three dimensional pattern (Fig. 2). All the individual elements or strands formed a sponge-like matrix. Only strands of a similar thickness could be clearly discerned. These strands were approximately 30 nm (nanometers) in diameter.

Examination of specimens prepared with digitonin for field emission scanning electron microscopy appeared as in Figure 3. The overall three dimensional arrangement of the cytoskeleton compares to and confirms the 3D structure observed in TEM, as shown in Figures 1 and 2. Long, very thin filaments appear to form a meshwork between the other filaments. Measurements of various filament diameters resulted in sizes ranging from about 10 nm to 40 nm, with some structures being 90 nm wide. The filaments could not be classified into three distinct sizes, according to the accepted classification of microfilaments of 6 nm, intermediate filaments of 10 nm and microtubules of 25 nm. Bundles of filaments also appeared to form major, structural components. These looked similar to stress fibres clearly identified in Figure 1 and present in Figure 4.

Thin filaments were also found on the surface of the nuclear membrane (Fig. 5). These filaments measured about 30 nm in diameter. Several nuclear pores are clearly shown, being defined by eight globular, protein sub-units arranged in a ring around the central pore.

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**Fig. 2.** 125 kV Transmission stereo pair showing a portion of the specimen in Figure 1. The intracellular, trabecular mesh appears to be a three dimensional network made up of filaments some 30 nm in width. The dense body in the matrix may be an intracellular organelle. ×45,000
Fig. 3. High resolution FESEM stereo pair of the intracellular structures resulting from digitonin detergent extraction of human fibroblast cells. The network consists of components having various diameters, but resembles the structure described by Porter and Tucker (1981) as the microtrabecular lattice. ×13,500

Fig. 4. High resolution FESEM stereo pair of intracellular network resulting from digitonin extraction of human fibroblasts. The network also contains larger fibres, apparently made up by bundles of smaller fibres (arrows). ×31,500
DISCUSSION

The methods for directly observing small volumes of whole cell mounts were developed by Porter and collaborators (Buckley and Porter, 1975; Wolsomewick and Porter, 1976; Porter and Stearns, 1981), who used high voltage 1000 kV TEM (HVTEM) to obtain stereo images of cells grown in culture on formvar films. It is also possible to obtain similar images of thinner parts of a cell using accelerating voltage of 125 kV, as shown in Figures 1 and 2. The micrographs obtained by using this substantially lower accelerating voltage showed that a three dimensional meshwork very similar in appearance to HVTEM results appears to exist within the confines of the cell. This meshwork has a characteristic arrangement which is easily recognized in suitable micrographs recorded by TEM. Because the patterns of meshwork may be obtained consistently and are repeatedly observed, it has led us to conclude that this meshwork is in fact a real structure present in the cell.

However, the influence and effects of various fixation and stabilization procedures inflicted on the cell cannot be ruled out. Processing methods could certainly account for inducing artificial structure and producing artifacts. How are these artifacts to be interpreted?

Two different fixation methodologies are presently being used to try and obtain 3D information about the cytoskeleton. One approach is to use various cryofixation techniques in order to avoid the use of chemical fixatives (Heuser and Kirschner, 1980; Pawley and Ris, 1987), the other methodology uses various chemical processes, but with an understanding of the effects and artifacts which may be introduced (Bell et al., 1989). Both methodologies may create new and unknown structures (Miller et al., 1983). As a consequence of the variations in cell structure as reported in the literature, several investigators (Heuser and Kirschner, 1980; Pawley and Ris, 1987) have suggested that the cytoplasmic meshwork seen by TEM and HVTEM is an artifact, likely produced by specimen preparation processes.

Based on their HVTEM results, Porter's group described this cytoplasmic meshwork as being a three dimensional network of tapering filaments and varying thickness that appear to link the cytoskeleton to intracellular organelles and possibly with the cell membrane. They concluded that the meshwork was formed from the cytomatrix by the cell, and
subsequently named this cytoplasmic meshwork the “microtrabecular lattice” (WOLOSEWICK and PORTER, 1979).

Based on these descriptions, close inspection of Figures 1, 2, 3 and 4 leads to the conclusion that we have imaged, by stereo FESEM, an intracellular, three dimensional meshwork which looks very similar to the microtrabecular lattice as described by PORTER. Figure 3 is very similar to a rendered interpretation of the microtrabecular lattice as published by PORTER and TUCKER (1981). Our FESEM results indicate that the microtrabecular lattice may indeed be a dynamic lattice, and that our method of preparation has preserved the cell at a stage where the lattice happened to have been maximally expressed.

We used digitonin in our fixation procedure in order to obtain the most advantageous fixation of the cytoskeletal components. It had previously been shown that digitonin, although a detergent of the Saponin type (FISKUM et al., 1980), extracted lesser amounts of intracellular matrix proteins while preserving the structure of cytoskeletal elements and mitochondria in an active state (KATZ and WALS, 1985; ROMERT et al., 1990; MITSUSHIMA and KATSUMOTO, 1990).

Earlier work by HENDERSON and WEBER (1979) using Triton X100 showed that microfilaments and microtubules obtained by this process compared favourably to our results for the three dimensional intracellular structure.

KATSUMOTO et al. (1983) used Saponin, another mild detergent, to produce holes in the membrane by removing cholesterol as well as removing soluble cytoplasmic proteins. Saponin may be destructive to the mitochondrial membrane but tends to spare cytoskeletal elements, lysosomes and endoplasmic reticulum.

We found that digitonin extracted less cytosolic protein than other detergents while maintaining the cytoskeleton and membranous organelles such as the nuclear envelope. However, some vesiculation of organelles, especially the endoplasmic reticulum was observed.

REFERENCES


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