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Effects of Cytochalasin B and Colchicine on the Morphology of SW-13 Human Adrenal Cortical Tumor Cells in Culture

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Abstract

Human adrenal cortical tumor cells (SW-13) grow into a typical epithelial cell monolayer when seeded onto culture dishes. The cells of the SW-13 population monolayer appear flattened with few conspicuous surface features. The cells are attached to one another at their lateral borders and are arranged in a cobblestone-like manner. Following Triton X-100 extraction, the distribution of the cytoskeletal elements was observed with scanning electron microscopic techniques to correspond to the shape of the non-extracted cell. Changes in the distribution and morphology of projections on the cell surface as well as changes in cell shape were revealed after treatment of the cultures with compounds which bring about microtubular and microfilament disruption. Following 60 minute treatment of the cell population with cytochalasin B (10μg/ml), 90% of the cells became round while remaining attached to neighboring cells and to the substrate by slender cell processes and filopodia. Some blebbing could be seen on the cell surfaces of cytochalasin B treated cultures and an increase in the number of microvilli was evident. When the cytoskeletal elements were observed with scanning electron microscopic techniques after Triton X-100 extraction, the amount of peripheral cytoskeletal elements was decreased and only slender projections of the microfilaments and microtubules were evident. Colchicine (0.06μg/ml) treatment of the SW-13 adrenal cell population resulted in the appearance of surface blebs within 10 minutes of the initiation of treatment. The changes in surface projections are discussed in relationship to the loss of microtubules and microfilaments from the cytoplasm of the cell.

Key Words: Cell Shape, Cytoskeletal Elements, and Adrenal Tumor Cells.

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Introduction

Microfilaments and microtubules have been demonstrated with transmission electron microscopy to be associated with the plasma membrane (Albertini and Clark, 1975; Pollard and Korn, 1973, reviewed by Schliwa, 1986). Actin and tubulin, two protein components of the microfilament and microtubule, have been demonstrated with biochemical techniques to co-purify with isolated plasma membrane, thus suggesting that microfilaments and microtubules may regulate the mobility and/or topographical distribution of cell surface components, as well as be involved in the morphology of the cell surface, (Edelman et al., 1973; Gruenstein et al., 1975; Bhattacharyya and Wolff, 1975). This study encompasses an investigation of the effect of colchicine, known to destroy microtubules, and cytochalasin B, a microfilament inhibitor, in SW-13 human adrenal cells in culture.

While the effect of cytochalasin B and colchicine on cell morphology has been demonstrated in many cells in culture (Godman, et al., 1975; Nicholson, et al., 1976; Rabinovitch and DeStefano, 1976; Tannenbaum, 1978; Weinreb, et al., 1986), the effect of these agents on SW-13 human adrenal cortical tumor cells has never been demonstrated. In this paper we have demonstrated the arrangement of the cytoskeletal elements with transmission electron microscopy and with scanning electron microscopy of extracted cells. This study presents for the first time ultrastructural information on the cytoskeletal elements of the SW-13 human adrenal cortical tumor cells. The SW-13 cell is an excellent cell system for the study of cancer etiology since it has been demonstrated to mimic the metastatic process in that once the cell population becomes confluent, cells are released into media and continue to proliferate above the monolayer (Murray, et al., 1981). In addition, this cell has been demonstrated to possess a relatively large number of cell-cell junctions in the monolayer and annular gap junctions in cells floating within the media (Larsen, et al., 1979 and Murray, et al., 1981).

The cells of this cell culture undergo changes in shape during the process of detachment

from the monolayer. We have investigated these shape changes and compared them to the shape changes that can be produced with cytochalasin B and colchicine treatment.

It has been suggested that important growth-related cellular functions such as proliferation (Folkman and Moscona, 1978; Iwig and Glaesser, 1985), the response to tissue-specific hormones (Gospodarowicz, et al. 1978), differentiation, (Benya and Shaffer, 1982; Glowacki, et al., 1983; Allen and Harrison, 1980), and tissue morphogenesis during embryogenesis (Hay, 1984; Spooner, 1975) can be modulated through changes in cell shape (reviewed by Ben-Ze'ev, 1985a,b and Bissell, et al., 1982). Changes in the shape of the cell are related to the distribution of the microtubules and microfilaments. The role of the microtubules and the microfilament in determining the shape of the cell, however, can differ in different cell types and the effect of colchicine and cytochalasin B on cell shape may have different effects in different cell types as well (Joseph-Silverstein and Cohen, 1984; Dustin, 1984; Weber and Osborn, 1982; Cohen and Terwillinger, 1979; and Behnke, 1970). The aims of the present study are to describe the presence of microtubules and microfilaments in the SW-13 cell and to demonstrate the role of cytoskeletal elements in maintaining the morphology of these cells in culture.

Materials and Methods

Cell Culture

Cultured SW-13 human adrenal cortical tumor cells were obtained from Leibovitz (Scott and White Clinic, Temple, TX). Cells were cultured in L-15 nutrient medium supplemented with cortisol (10μg/ml), insulin (0.01 units/ml), penicillin (0.06 mg/ml), streptomycin (0.1 mg/ml), fungizone (0.01 mg/ml) and 10% fetal calf serum, all purchased from Grand Island Biological Co. (Grand Island, NY). The cells were grown in 35 mm Falcon petri plates or 25 cm^2 Falcon flasks (Falcon Plastics, Oxnard, CA) at 37°C in a 5% CO_2 atmosphere.

Treatment of Cells

SW-13 cells were seeded at 5 x 10^4 cells/cm^2 into 25 cm^2 flasks (Falcon) or 35mm plates and allowed to grow to near confluency. Fresh medium at 37°C containing either colchicine (0.06 μg/ml) (Sigma) or cytochalasin B (10 μg/ml) (Sigma) was added to the cultures. The cells were incubated for 60 minutes at 37°C and then prepared for scanning, transmission and freeze-fracture electron microscopy.

Scanning, Transmission and Freeze-Fracture Electron Microscopy Studies

Cells for electron microscopy were seeded into 35 mm plates or 25cm^2 flasks and allowed to attach to the vessel. Following treatment, the cell cultures were fixed for 20 minutes in situ at room temperature in 2.5% glutaraldehyde and 0.06 M sucrose buffered to pH 7.2 with 0.05 M cacodylate buffer. Cells in the media were collected on a filter paper and the cells were then fixed while on the filter paper. After a change of fixative, the cells for scanning, transmission, and freeze fracture electron microscopy were rinsed in 0.05 M cacodylate buffer containing 0.06 M sucrose and postfixed in 1% osmium tetroxide buffered to pH 7.2 with 0.05 M cacodylate. Although the rinse solution was not isotonic, this did not result in blebbing of the cell membrane of control cells.

After a second buffer wash, the cells were dehydrated in a graded ethanol series. The cultures to be scanned were transferred to a critical point dryer (Tousimis Research Corp., Rockville, Maryland), and dried through CO_2. The preparations were then coated with gold-palladium in a Hummer V sputter coater (Technics Corp., Alexandria, Virginia). The cells were scanned in either a Cambridge S4 or a JEOL T300 scanning electron microscope.

The cells for transmission electron microscopy were dehydrated in ethanol, embedded in araldite (R.P. Cargille Labs, Inc., Cedar Grove, NJ), and sections were cut with the Huxley LKB microtome. Cultures used in freeze fracture studies were infiltrated with 30% glycerol for 3 hours after 10 minutes of fixation in 2.5% glutaraldehyde and 0.06 M sucrose in 0.05 M cacodylate buffer at pH 7.2. The cells were frozen in Freon and fractured with a mirror image device in a Balzers BA E 121 freeze-fracture apparatus (Balzers, Hudson, NY). Complimentary platinum-carbon replicas were cleaned and mounted on 300 mesh grids. Micrographs were taken with a Phillips 300 electron microscope.

Preparation of Cytoskeleton for SEM - Detergent Extraction

The cytoskeleton was prepared for scanning electron microscopy with a Triton X-100 extraction procedure (Bell and Stark-Vancs, 1983). The 5 x 10^4 cells/cm^2 were seeded into 35 mm plates on glass coverslips and allowed to attach to the substrate. After treatment, the culture medium was removed from the tissue culture plates and the cells were rinsed once in phosphate buffered saline at pH 7.4 and 37° C. The cells were extracted by adding 1 ml of the extraction medium containing 0.5% Triton X-100, 1 mM ethylene glycol-bis (-aminoethyl ether) N, N'-tetraacetic acid (EGTA), 4% polyethylene glycol (PEG) 4000, 100 mM piperazine-N, N'-bis (2-ethanesulfonic acid) (PIPES) at pH 6.7 for 8 minutes at 37° C. The extraction medium was gently removed and the cytoskeletons were fixed in 2.5% glutaraldehyde in 0.1 M sucrose, 0.1 M cacodylate, pH 7.4, for 30 minutes at 37° C. The coverslips were then rinsed three times in 0.1 M sucrose, 0.1 M cacodylate buffer, pH 7.4, and postfixed in 1% OsO_4 in the same buffer at pH 7.4. The coverslips were then dehydrated to 100% ethanol, critical point dried from CO_2 in a critical point drying apparatus (Tousimis Research Corp., Rockville, Maryland) and coated with gold-palladium in a Hummer V sputter coater (Technics Corp., Alexandria, Virginia). The coverslips were examined in a JEOL T 300 scanning electron microscope.

Results

Morphology of the Control Cell Population

The cells of the SW-13 monolayer, when

viewed with scanning electron microscopy, appeared flattened with few conspicuous surface features (Fig. 1). No blebbing of the cell surface was detected. The cells of the monolayer were attached to one another at their lateral borders and were arranged in a cobblestone-like manner. The Triton-extracted cell outline was formed by a cytoskeletal fibrous material that closely approximated the outline of the original cell viewed with scanning electron microscopic techniques (Fig. 2). The cytoskeletal fibers formed a dense but open network with numerous spaces in the meshwork. Areas where there were large spaces between the individual fibers were also apparent. The fibers varied in size. The cell nucleus was centrally located and some fibers could be seen over the nucleus. Individual fibers extending as individual linear elements could be seen at the edge of the cell. Spheres of various sizes were occasionally seen in the Triton-extracted cells. These spheres were embedded in the cytoskeletal matrix and had smooth surfaces. The spheres were thought to represent the remains of cytoplasmic structures such as lysosomes and residual bodies that were not washed out of the cells after the Triton extraction treatment.

When viewed by transmission electron microscopy, SW-13 cells were relatively uniform in shape and bundles of microfilaments and microtubules could be observed in the peripheral cytoplasm (Figs. 3,4). The nuclei, with densely staining chromatin, were centrally located and the mitochondria were long and cylindrical with lamellar cisternae. As the SW-13 cell monolayer became confluent, the flattened cells were observed to round but remained attached to the substrate by short, slender processes (Murray, et al., 1981). Shortly after rounding, detachment of these fibers from the substrate occurred and the rounded cells became free to float in the medium. Once in the medium, they either divided and respread on the substrate or the rounded cells became attached to the top surface of the monolayer. In the latter case, they either remained single, or with time, spherically aggregated with other cells to form large aggregates or domes (Murray et al., 1981). The rounded cells were observed to be scattered throughout the surface of a confluent monolayer population.

The surface of the rounded cell, as revealed with scanning and transmission electron microscopy, was either smooth or covered with blebs (Figs. 5,6). The blebs covering the cell, when viewed with transmission electron microscopy, lacked cytoskeletal elements but contained many irregularly sized vesicles outside the region of blebs. The cytoplasm within the area of the bleb had fewer microtubules and microfilaments (Fig. 6). The nucleus was rounded and centrally located in both the rounded cells and the cells of the flattened monolayer. A relatively large percentage (88%) of these rounded cells possessed mitotic figures. The large number of mitotic figures is supportive of the work of other investigators who have suggested that rounded mitotic cells are released into the medium and divide while in the floating state (Terasima and Tolmach, 1963). In some cases, cell debris and dead cells were also observed floating above the monolayer.

Morphology of Experimentally Treated Cell Populations.

Treatment of the cell population with 10μg/ml cytochalasin B for 60 minutes produced morphological alterations which could best be characterized with scanning electron microscopy. The surface of the cytochalasin B treated cell, unlike the smooth upper surface of the control monolayer population (Fig. 1), was characterized by blebs, and by long fibers which projected from the cell body (Fig. 7). The cells remained in contact with one another as well as with the substrate by slender projections, giving the cells an arborized appearance. The cell body became rounded and the cell morphology changed from that of a circular, radially-spread contour to a more polarized state. When the cell was viewed after Triton extraction, the cytoskeleton was seen to have become more dense in the area surrounding the nucleus (Fig. 8). The arborized outline of the cell was apparent and the cytoskeletal material remained extended to persisting adhesion sites. Unlike the observations in BKH cells (Bell 1981) in which the nucleus area was not overlain by cytoskeletal fibers following extraction, cytoskeletal elements covering the SW-13 nucleus remained after the extraction of cytochalasin B treated cells. The cytoskeleton appeared to be contracted around the nucleus. The cytoskeletal fibers were less densely packed in the periphery than in the control cultures. In contrast to the changes seen after cytochalasin B treatment, colchicine (0.06 μg/ml) treatment of a confluent monolayer of cells for 60 minutes resulted in surface blebbing and retraction of the cells from one another along their borders although the cells remained attached to the substrate and to one another by broad projections (Fig. 9). The surface blebs following colchicine treatment were smaller and more numerous than the blebs observed in rounded cells found in the media above the monolayer of control cultures. Transmission electron microscopy revealed that the blebs were filled with many vesicles but lacked other cytoplasmic organelles. The outline of the colchicine treated cell observed following Triton extraction was similar to that of the control cultures (Fig. 10). The cytoskeletal meshwork is more dense than that of the cytochalasin B treated culture (Fig. 10). Numerous spheres of various sizes were seen in the Triton-extracted, colchicine treated cells (Fig. 10). These smooth surfaced spheres were thought, as in the control, to represent the remains of structures that were not washed out of the cells after extraction.

The Effect of Cytochalasin B and Colchicine on Membrane Structure

With electron microscopy two types of membrane specializations were identified. The one resembled "intermediate junctions" described by other investigators (Perissel, et al., 1976) and had a 20 nm space between the two membranes and a dense amorphous material on the cytoplasmic

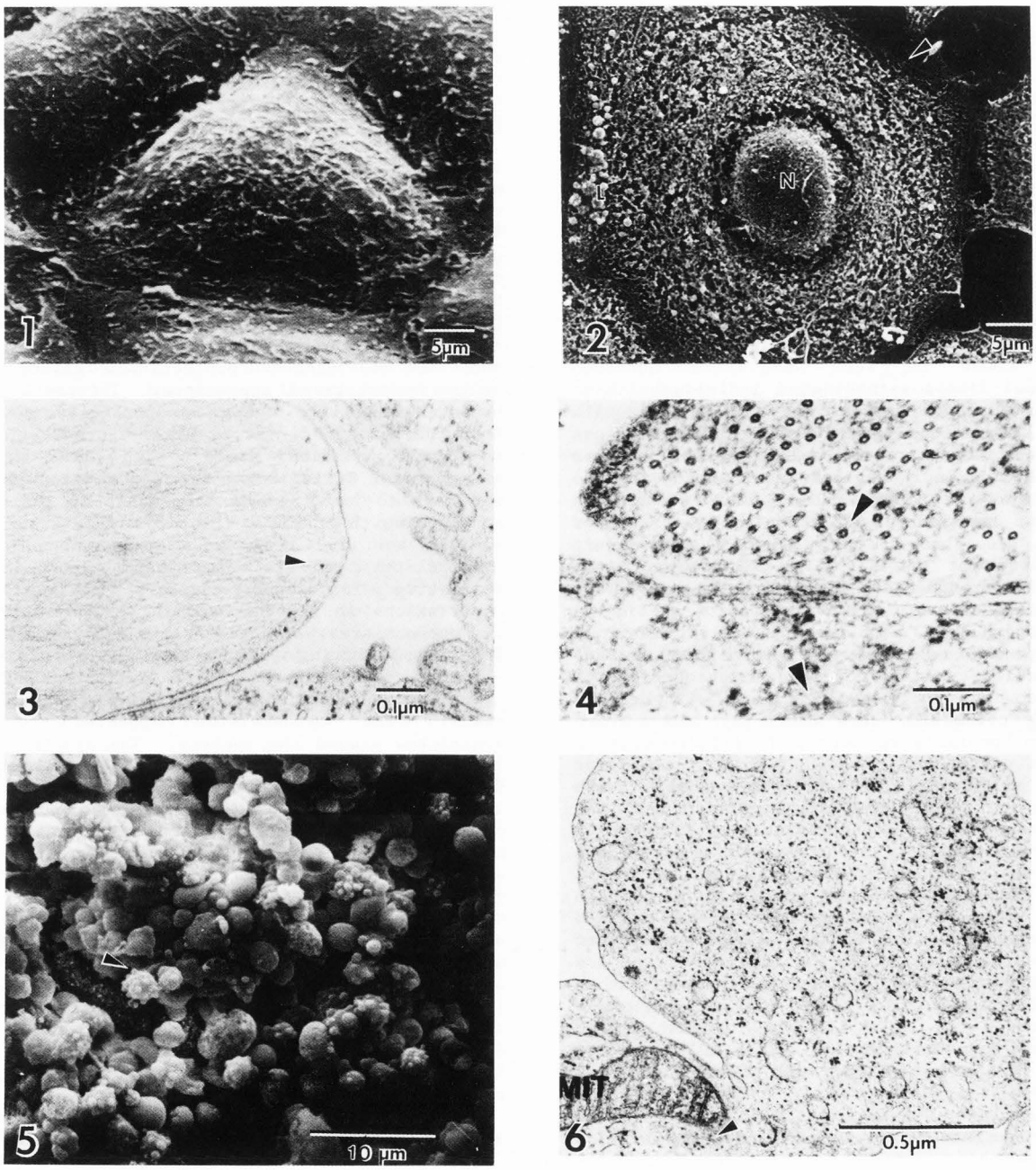

Fig. 1. Scanning electron micrograph of an SW-13 adrenal cortical tumor cell population showing the flattened cobblestone-like cells of the monolayer.
Fig. 2. Scanning electron micrograph of an SW-13 human adrenal cortical tumor cell after Triton extraction. Note the cell nucleus (N), the arrangement of the microfilamentous cortex (arrow), and lipid droplets (L).
Fig. 3,4. Thin section electron micrographs of an SW-13 human adrenal cortical tumor cell population showing a bundle of microfilaments and microtubules (arrow) in the peripheral cytoplasm.
Fig. 5. Scanning electron micrograph of a cluster of spherical SW-13 cells collected from the medium above the monolayer on a millipore filter. The surface of some spherical cells have many blebs (arrow).
Fig. 6. Thin section micrograph of an SW-13 cell showing vesicles and ribosomes in the cytoplasm of one of the surface blebs of a spherical cell. Note the absence of microtubules and microfilaments within the bleb. (Arrow points to microtubules, and MIT identifies a mitochondrion.)

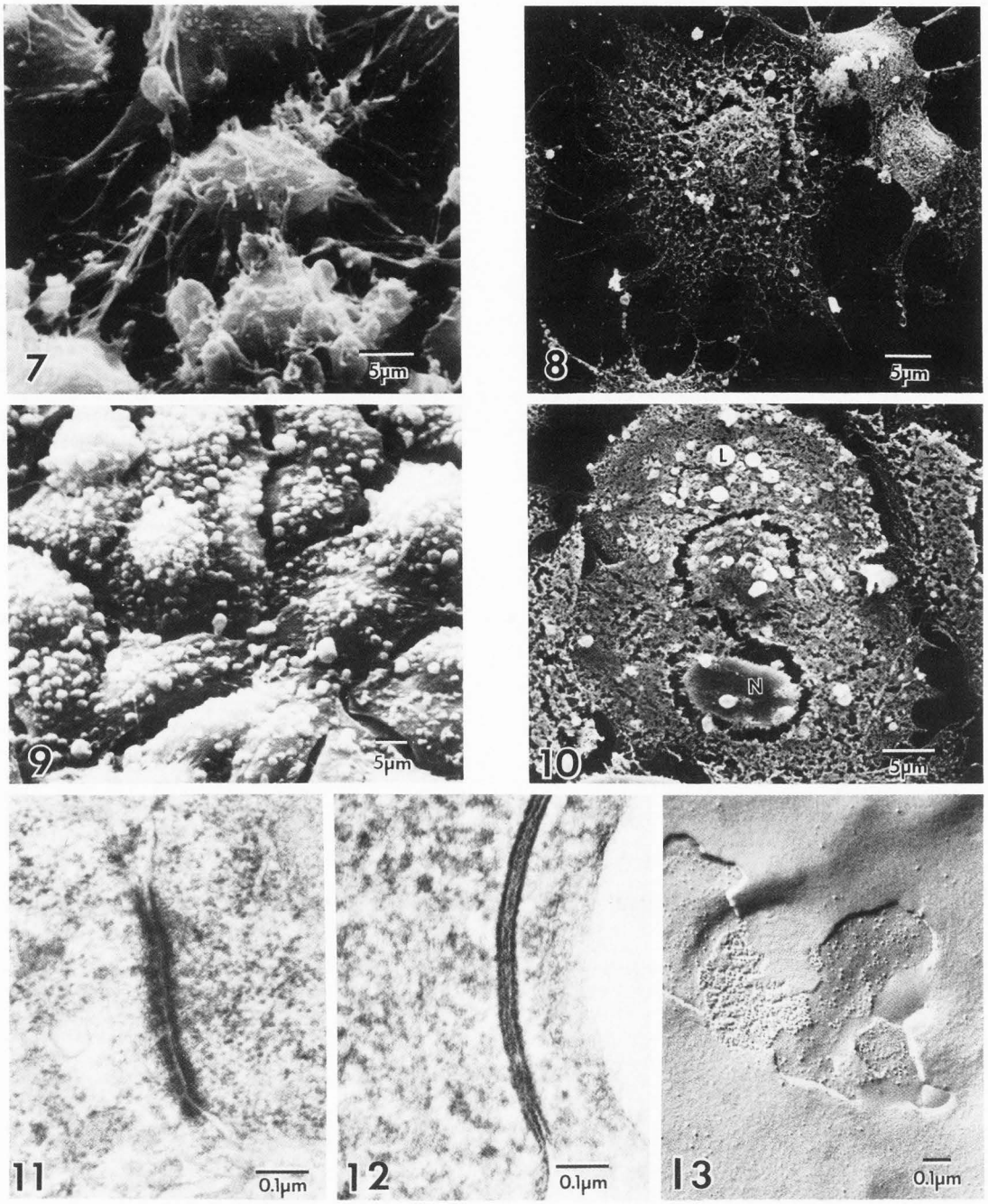

Fig. 7-10. Scanning electron micrographs of the SW-13 monolayer of cells.
Fig. 7. Culture treated for 60 minutes with 10 μg/ml cytochalasin B. Note the arborized appearance of the cell population, the presence of surface blebs and long cell processes.
Fig. 8. Culture treated for 60 minutes with 10 μg/ml cytochalasin B and then Triton-extracted. Note the retraction of the cytoskeletal elements from the periphery.
Fig. 9. Culture treated for 60 minutes with 0.06 μg/ml colchicine. Note the surface blebbing.
Fig. 10. Culture treated for 60 minutes with 0.06 μg/ml colchicine and then Triton-extracted. Lipid droplet, (L), Nucleus, (N).
Fig. 11. Thin section micrograph of an SW-13 cell intermediate junction.
Fig. 12. Thin section micrograph of an SW-13 cell gap junction.
Fig. 13. Freeze fracture electron micrograph of SW-13 cell membrane showing the typical distribution of particles on the membrane. A cluster of particles forming a gap junction can be seen.

surface (Fig. 11). Microfilaments ran into a filamentous mat which was closely applied to the cytoplasmic side of the opposed plasma membranes. These junctions lack the large cytoplasmic plaque and the intercellular filaments which are typical of desmosomes. The second membrane specialization was the gap junction. This junction was characterized by the 2-3 nm gap between the outer leaflets of the junctional membrane section (Fig. 12). The gap junctions varied in length from 0.1 μm to 1.5 μm when viewed with transmission electron microscopy. Ultrastructural observations of the surface architecture of freeze-fractured SW-13 cells revealed a random distribution of intramembranous particles approximately 8-10nm in diameter. The majority of particles were singularly distributed over the P fracture face of the membrane. With freeze-fracture techniques, gap junctions were characterized by the presence of 8-10 nm particles tightly aggregated on the P fracture face and pits on the E fracture face (Fig. 13). Gap junctions were observed on the membranes of control cells as well as on the membranes of cells treated with colchicine, and cytochalasin B.

Discussion

Cytochalasin B treatment in this study resulted in an arborization of the culture. It can be suggested that the flat cell morphology seen in the control culture is maintained by the presence of these microfilament bundles. In contrast, colchicine treatment of the SW-13 cell population did not result in an overall change in cell shape as seen following cytochalasin B treatment, but did yield a change in surface morphology characterized by an increase in surface blebbing. It was observed with thin section electron microscopic techniques that these surface blebs were relatively free of organelles and that there was a decrease in the presence of microtubules. Since colchicine is thought to bind to the growing microtubule during assembly such that further assembly is blocked, the increase in surface blebbing seen in the colchicine treated SW-13 cells would indicate that microtubules play a role in maintaining the smooth surface morphology seen in the control population. Pronounced peripheral densities have been reported on the surface of colchicine treated cultures by other investigators (Albertini and Anderson, 1977; Weinreb, et al., 1986; Keller, et al., 1985). With SEM techniques the nature of such densities can be revealed and presumably are these blebs.

While it is realized that the use of colchicine in this study may produce many non-specific effects, colchicine has, however, proven effective in inhibiting microtubules by inhibiting polymerization of tubulin by combining with the tubulin dimer (Wilson, et al., 1974, Detrich, et al., 1982; Andreu and Timasheff, 1982.). Since the dimers cannot polymerize, the existing microtubules disappear and the cell functions that are dependent on the presence of microtubules are inhibited. In our study, change in shape following colchicine was fairly rapid with the full effects being visualized following 60 minute treatment. Disruption of the microtubules with colchicine did not result in a loss of cell-substrate contact. The SW-13 cells remained flat and well spread on the substrate.

Cytochalasin B inhibits the polymerization of actin by binding to the ends of microfilaments (Lin, et al., 1980). Cytochalsin B, in addition, causes the shortening and reorganization of the microfilaments (Lin, et al., 1980: Mac Lean-Fletcher and Pollard, 1980). The observation of membrane blebbing following cytochalasin B treatment in our study is consistent with the observations that microfilaments may attach to transmembrane proteins that penetrate the membrane and perhaps even bind to extracellular structures (Bissell, et al., 1982; Jockusch and Isenberg, 1981). If this were the case, then disruption of microfilaments would result in a detachment of the cell membrane and a decrease in the restraining influence provided by the microfilaments. Thus, blebbing would result. The observation of blebbing following colchicine treatment would also suggest an effect, either directly on the cell membrane or via interactions with the microfilaments on the cell membrane by the microtubules to provide a restraining force.

Since the cytoskeletal elements play a pivotal role in maintaining cell shape, it was important to investigate how inhibitors of cytoskeletal elements affected the morphology of a slow growing adrenal cortical tumor cell. During cell transformation, various alterations occur in the organization of the cytoskeleton. The elucidation of the involvement of cytoskeletal elements in control of cell shape will increase our knowledge of cell morphogenesis as well as be a step in the understanding of how shape changes may function related to alterations in cellular function and proliferation. The observations in this study represent a preliminary characterization of the changes in SW-13 cell morphology following cytoskeletal element disruption. This cell system may serve as a good model for the further study of gap junction formation and turnover as well as serving as a model for further study of the relationship of morphology to microtubules and microfilaments.

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Discussion with Reviewers

L. Phaire-Washington: Is the morphological damage produced by cytochalasin B and colchicine reversible?

Authors: We have allowed the cells to recover after the cytochalasin B (10μg/ml) treatment and then observed them with the phase microscope. When the cells were removed from cytochalasin B, rinsed twice with Earle's balanced salt solution, and then returned to cytochalasin B free medium, they flattened and respread on the substrate such that the arborized appearance was lost. In addition, cells that were lifted from the monolayer by vigorously shaking the cytochalasin B treated culture respread on the substrate and formed a typical epithelial monolayer when reseeded into fresh medium. At higher concentrations of the cytochalasin B, however, the damage done by the drug can be so extensive that the cells do not recover from the treatment.

We have not studied the recovery of this cell population after colchicine treatment. The colchicine treated cultures were terminated at the end of one hour and used for either scanning, transmission or freeze fracture electron microscopy.

L. Phaire-Washington: How do you know the amount of peripheral cytoskeletal elements was decreased in drug-treated cells? Is there biochemical evidence of a decrease in the concentration, or were ratios of polymerized vs. unpolymerized proteins assessed morphologically? Were you able to quantitate the amount of soluble and insoluble cytomatrix proteins after 60 minutes of drug treatment?

Authors: We made no attempt to quantitate the amount of soluble and insoluble cytomatrix proteins after 60 minutes of drug treatment. We have, after Triton X-100 extraction, noticed what appeared to be a reduction in the amount of peripheral material. We have examined a number of cell populations and found that the cytomatrix looked sparse in the drug-treated cells when compared to the control cultures.

L. Phaire-Washington: Because of the myriad of side effects of cytochalasin B, did you investigate morphological effects of D and E which have more direct effect on the actin-rich microfilaments?

Authors: We have not investigated the morphological effects of the cytochalasin D or E on the SW-13 adrenal cell. It is worth pointing out, however, that the cytochalasin D and E, as stated by the reviewer, have a more direct effect on microfilaments than cytochalasin B. Cytochalasin B not only disrupts the actin filaments but also has an effect on a number of other parameters including glucose uptake. We have not, however, investigated the morphological effects of the D or the E, but such studies are in progress.

L. Phaire-Washington: Is the cytoskeletal organization the same as in rounded, non-colchicine treated cells as shown in figure 5 and 6?

Authors: In the control, as in the treated cultures, the blebs contained numerous vesicles but lacked other cytoplasmic organelles. The cytoplasm within the area of the bleb of both the rounded non-colchicine and the colchicine treated cells had fewer microtubules and microfilaments than flattened cells of the control monolayer. Since the ultrastructure of the blebs appeared similar in the treated and in the control cultures, we have not included a picture of the blebs from each of the treatment groups. Instead, we have chosen to only include a micrograph of a bleb from a control culture (figure 6).

L. Phaire-Washington: What effect did cytochalasin B have on the ultrastructure of intermediate junctions (figure 11)? You stated that microfilaments ran into a filamentous mat which was closely applied to the cytoplasmic side of the opposed plasma membranes. How did colchicine or cytochalasin B alter the morphology of these junctions?

Authors: We found examples of intermediate junctions with the typical filamentous mat in the colchicine, cytochalasin B and in the control cultures. The morphology of the intermediate junction did not appear to be affected by either colchicine or cytochalasin B treatment. We have not yet attempted to quantitate the frequency of these junctions following drug treatment.

L. Phaire-Washington: There appears to be filamentous mesh like material within vesicles in the cytoplasm of surface blebbing areas of a spherical cell. Do you have any information as to contents of the vesicles? Is it possible that much of the cytoplasmic microfilaments are autophagocytosed and compartmentalized within such membranous vesicles?

Authors: We do not know the nature of the

content of the cytoplasmic vesicles. We have not made an attempt to identify the material in the vesicles of rounded cells. We do not, however, think that the microfilamentous material is being internalized into these vesicles. Our investigations would not permit such a conclusion and we are unaware of such a autophagocytosis of the microfilament following drug disruption.

L. Phaire-Washington: Do you have correlative freeze-fracture information on cytochalasin treated cells? How does cytochalasin affect distribution of the particles around the gap or intermediate junctions?
Authors: The particles of the intermediate junction have not been described in the literature with freeze fracture techniques. The gap junction particles and their distribution have, however, been described in the SW-13 following freeze fracture technique (Murray et al., 1981). We have not quantitated the number or the fractional area covered by gap junctions following either colchicine or cytochalasin B treatment. We have, however, observed the presence of gap junctions as well as forming gap junctions (formation plaques) following both colchicine and cytochalasin B treatment. There were no apparent alterations in the morphology of the gap junctions as observed with freeze fracture following either of the treatments. Other investigators have suggested that intact microfilaments and microtubules are not required for gap junction formation (Kidder et al., 1987). Furthermore, treatment with colchicine and with cytochalasin B did not prevent the development or maintenance of gap junctional communication (Ito et al., 1974).

L. Phaire-Washington: What is the evidence that the circular material in fig. 2 are lipid droplets?
Authors: The spheres of various sizes found embedded in the cytoplasm are probable detergent-insoluble lipid droplets, as described by Bell (1981). He has stated that spheres in other Triton-extracted cells are osmophilic, extracted by organic solvents, and stainable with lipid-specific dye oil red O. We have not, however, stained these cells for lipid specific dye red O and can therefore suggest only that they are inclusions found within the cell.

P.B. Bell: The spheres of various sizes found embedded in the cytoskeleton were suggested to be lysosomes or other organelles. It is my opinion that these are detergent-insoluble lipid droplets. This conclusion is based upon my observation of similar spheres in other Triton-extracted cells that are osmophilic, extracted by organic solvents, and stainable with the lipid-specific dye oil red O. Would you please comment on this possibility?
Authors: We have not done staining for lipid-specific dye oil O and, therefore, cannot comment with certainty on the nature of these spheres. However, the spheres do resemble, in size, the lipid droplets sometimes found in the cytoplasm.