

MICROFILAMENTS IN EPITHELIAL MORPHOGENESIS

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INTRODUCTION

Cytoplasmic microfilaments have recently been implicated as the contractile organelles responsible for some of the changes in cell shapes and in intercellular organization occurring during epithelial morphogenesis in embryonic development (7). The experiments reported here indicate that strikingly similar coordinated cellular changes can be induced *in vitro* in monolayers of some established epithelial cell lines, that these changes occur in the absence of any heterologous tissues, and that they, too, seem dependent on the integrity of contractile cytofilaments.

The cell line C-4II, used here, was originally derived from a squamous-cell carcinoma. Over years in culture, cells of this line retained some of the characteristics of normal epithelial basal cells, including the ability to form simple multicellular structures *in vitro*, and to form basement membranes *in vivo* (1). It was demonstrated previously that fragments of confluent monolayers of line C-4II become convoluted in response to removal from solid substrata (2), and that the subcellular changes accompanying this process resemble those described for contracting epithelia *in vivo* (4). The dependence of this convoluted on the integrity of the cytoplasmic microfilaments has now been tested with cytochalasin B, a substance thought to interfere specifically with the function of these organelles (5).

MATERIALS AND METHODS

Cultures of line C-4II were maintained in plastic Petri dishes in Waymouth's medium MB 752/1 with 10% fetal calf serum, 100 U/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin. When monolayers were confluent (Fig. 1), fragments of cell sheets, up to 1–2 mm in diameter, were detached from the plastic substratum with tips of Pasteur pipettes, collected by

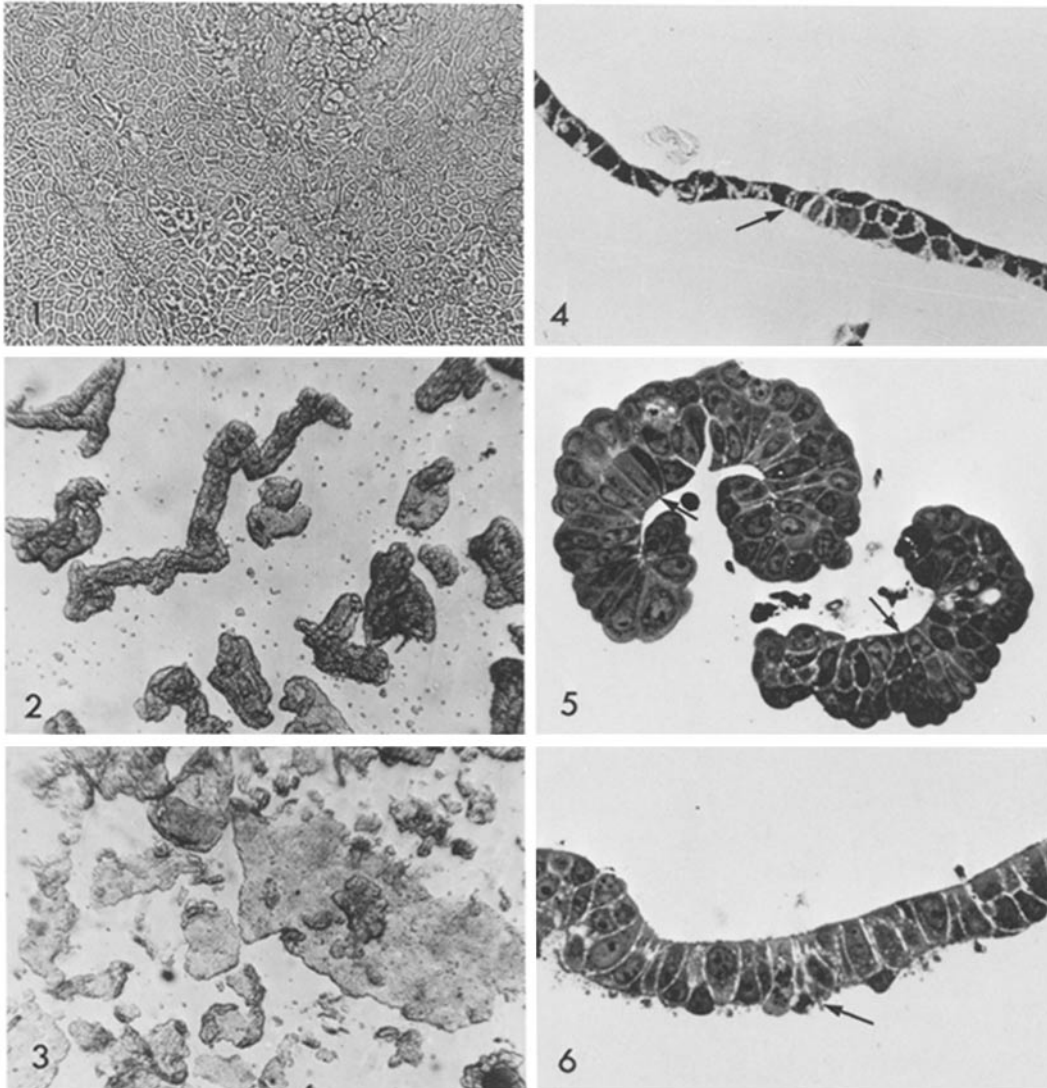
brief centrifugation, and resuspended in fresh culture medium that contained either no additives (control medium) or one of the following: (a) 10.0 $\mu\text{g}/\text{ml}$ cytochalasin B and 1.0% dimethyl sulfoxide (DMSO); (b) 1.0 $\mu\text{g}/\text{ml}$ cytochalasin B and 0.1% DMSO; (c) 1.0% DMSO; (d) 0.1% DMSO. Cytochalasin B was added to the culture medium as a stock solution of 1.0 mg/ml in DMSO as suggested by Carter (3). The suspended cell sheets were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, in Petri dishes that had been coated with 2% purified agar (Difco Laboratories, Inc., Detroit, Mich.) to prevent readhesion of the cells.

After incubation for periods ranging from 30 min to 24 hr, the cells were fixed in 2.5% glutaraldehyde/Millonig's buffer at room temperature for 30–60 min, postfixed with 1% OsO₄, and further processed for electron microscopy as described previously (1). For light microscope examination of fixed material, sections 0.2–0.5 μ thick were stained with 1.0% toluidine blue/1.0% borax.

RESULTS

Within minutes after suspension in the control medium, the fragments of C-4II monolayers began to roll up, and by 2 hr they had formed tightly convoluted tubular structures (Fig. 2) that remained essentially unchanged over the next 20 hr. No difference between this control group and cultures suspended in either 1.0% DMSO or 0.1% DMSO could be observed, and there were only inconclusive differences upon exposure to 1.0 $\mu\text{g}/\text{ml}$ of cytochalasin B. However, 10.0 $\mu\text{g}/\text{ml}$ of cytochalasin B inhibited the convoluted, so that the suspended cell sheets remained flat or slightly folded after detachment from the plastic (Fig. 3).

Cross sections through fragments fixed immediately upon suspension in control medium showed no change in their organization (Fig. 4) or ultrastructure (2). However, if the fragments were fixed after 2 hr of suspension, when the cell sheets were



FIGURES 1-3 Living cultures of C-411 cells, standard optics.

FIGURE 1 Confluent monolayer, growing on plastic substratum. $\times 120$.

FIGURE 2 Fragments of monolayer suspended for 2 hr in control medium. The cell sheets are convoluted. $\times 40$.

FIGURE 3 Fragments of monolayer suspended for 2 hr in medium with $10 \mu\text{g/ml}$ of cytochalasin B and 1.0% of DMSO. The convolution seen in Fig. 2 is greatly reduced. $\times 40$.

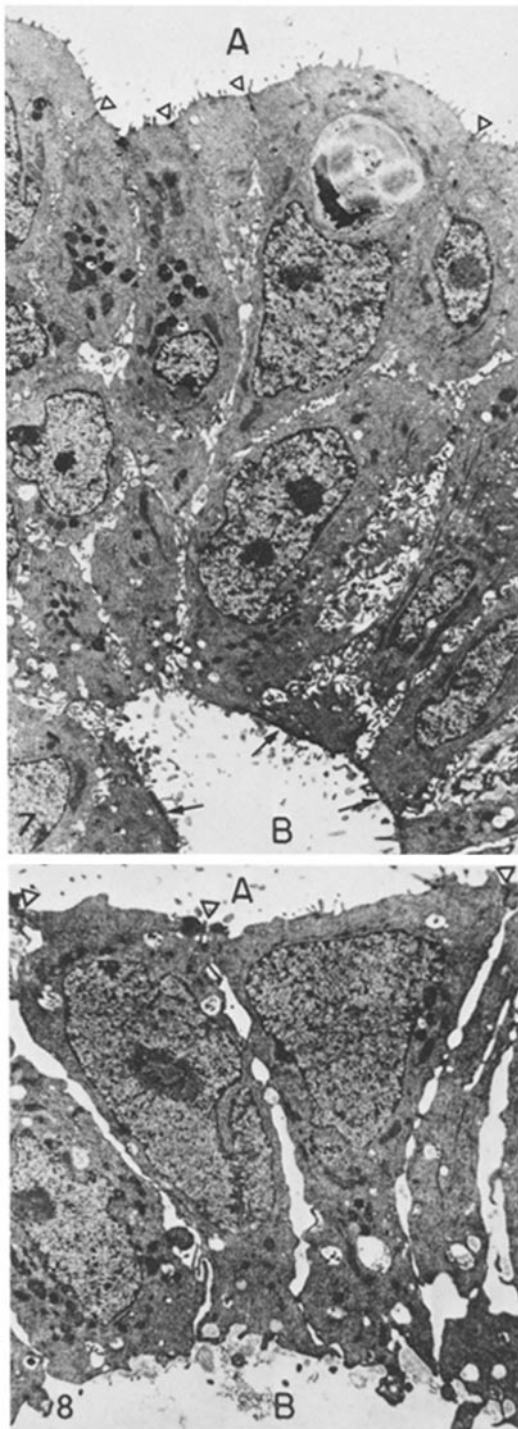
FIGURES 4-6 Vertical sections through suspended, fixed fragments of C-411 monolayers. Arrows point to the basal surface, i.e. to the side of the cell sheets that was attached to the substratum. Epon 812 embedding, Toluidine blue.

FIGURE 4 Fixation immediately upon detachment from the substratum. The cells remain organized in a columnar monolayer. $\times 440$.

FIGURE 5 Fixation after 2 hr of suspension in control medium (see Fig. 2). The basal surface of the cell sheets is contracted; the apical surface is scalloped. $\times 440$.

FIGURE 6 Fixation after 2 hr of suspension in medium containing $10 \mu\text{g/ml}$ of cytochalasin B and 1.0% of DMSO (see Fig. 3). The cell sheet is folded slightly towards the apical side. $\times 440$.

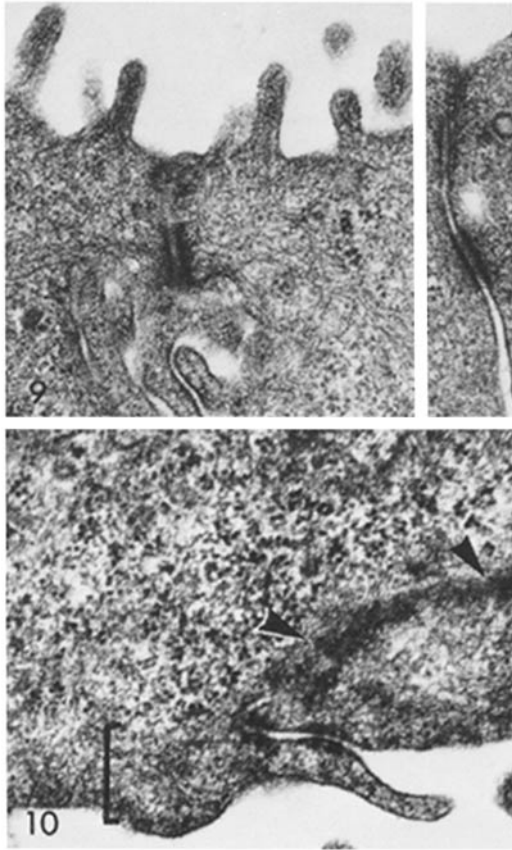
rolled up, the intercellular spaces were reduced, and the cells were elongated along the axis perpendicular to the former side of attachment to the



substratum, but were narrowed along the axis parallel to the same side (Fig. 5). This narrowing was greatest at the inner, concave surface of the convoluted sheets, suggesting that the cells had undergone coordinated localized contractions on this side. On the other hand, the outer, convex surface of the structures was scalloped, as if, in response to lateral pressure, the cells were bulging beyond fixed points of intercellular adhesion. Ultrastructurally, these points were in fact identified as junctional complexes, and the outer surface of the convoluted sheets was identified as the apical side of the cells, i.e. the side which had been exposed to the culture medium before their removal from the plastic. In addition to the junctional complexes, this outer surface had retained the characteristic prominent, regularly spaced microvilli, and the juxtaposed cortical cytoplasm contained a network of microfilaments reminiscent of a terminal web (1). Underlying this apical region of the cells, the nucleus, particulate organelles, microfilament bundles, and some microtubules were all oriented perpendicular to the former growth surface (Figs. 7, 9). The most striking ultrastructural change had taken place at the inner, contracted side of the convoluted sheets, i.e. at the former basal aspect of the cells. There, the plasma membranes were folded, and the adjacent cytoplasm contained a dense layer of 40 Å wide microfilaments. These filaments were oriented predominantly parallel to the basal cell surface and seemed to be continuous with dense junctions that connected all adjacent cells (Figs. 7, 10). (It should be noted that neither filament bands nor dense junctions were observed in C-411 monolayers fixed

FIGURE 7 Electron micrograph of section through cell sheet suspended for 2 hr in control medium (see Figs. 2, 5). At the contracted basal surface (B), the plasma membrane is folded, and adjacent to it there is a dense filament band (solid arrows). At the apical surface (A) the cells are joined by junctional complexes (∇) and form numerous microvilli. Most nuclei, particulate organelles, and perinuclear filament bundles are oriented vertically. $\times 3000$.

FIGURE 8 Electron micrograph of section through cell sheet suspended for 2 hr in medium with 10.0 $\mu\text{g/ml}$ cytochalasin B and 1.0% DMSO (see Figs. 3, 6). There is no microfilament band at the basal surface (B), intercellular spaces are wider than in Fig. 7, and the apical surface (A) shows microvilli and junctional complexes (∇), but is flat rather than scalloped. $\times 3600$.



FIGURES 9-10 Electron micrographs of cell sheet suspended for 2 hr in control medium (see Fig. 7).

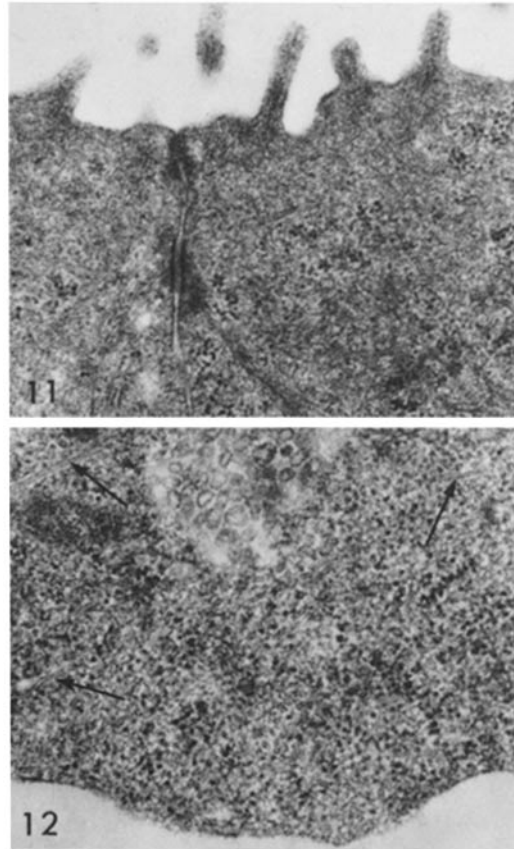
FIGURE 9 Apical cortical cytoplasm. Filaments of the subsurface network extend into microvilli; others are associated with a desmosome. *Inset*, Junctional complex from an adjacent area. $\times 29,000$.

FIGURE 10 Basal cortical cytoplasm. Region near the plasma membrane is taken up by a filament layer at the exclusion of other organelles (bracket). At the lower right, the section cuts tangentially across the filament layer of a neighboring cell, and through the condensation of filaments associated with the dense junction (arrows). $\times 30,000$.

either while attached to the substratum or immediately upon detachment therefrom [1, 2].)

In cultures fixed after 2 hr of suspension in 10.0 $\mu\text{g}/\text{ml}$ cytochalasin B, the cells had remained columnar, intercellular spaces were wider, and the apical surfaces of the cells were even rather than scalloped. The cell sheets were flat or folded slightly against the apical side (Fig. 6). The con-

spicuous basal filament bands and dense junction seen in suspended control cultures were absent. At the basal surface there were many pseudopodium-like protrusions which were often filled with granular material similar to that seen in other cytochalasin B-treated cells (Figs. 8, 12) (7). In contrast to these effects on the basal cytoplasm, the 2 hr treatment with cytochalasin B did not seem to disrupt the organization of the junctional complexes or of the microfilaments in the apical cortical cytoplasm (Fig. 11). It also did not have any



FIGURES 11-12 Electron micrographs of cell sheet suspended for 2 hr in medium with 10.0 $\mu\text{g}/\text{ml}$ cytochalasin B and 1.0% DMSO (see Fig. 8).

FIGURE 11 Apical cortical cytoplasm. Filaments of the subsurface network extend into microvilli. A junctional complex and associated filaments are present. Note similarity to Fig. 9. $\times 24,000$.

FIGURE 12 Basal cortical cytoplasm. The filament layer seen in Fig. 10 is absent. Ribosomes extend to the plasma membrane. Microtubules (arrows) appear intact. $\times 30,000$.

obvious effect on the appearance of those filaments that were arranged along the long axis of the cells, on microtubules, on apical microvilli, or on desmosomes.

DISCUSSION

The similarity in ultrastructural changes between the *in vitro* convolution of the C-4II sheets and normal epithelial morphogenesis under more physiological conditions (7) strongly suggests a common basic mechanism involving localized, coordinated cellular contractions. While, *in vivo*, the intracellular polarity in the distribution and/or function of microfilaments required for such contractions may be induced by interactions of the developing epithelia with heterologous tissues (6, 8), the behavior of the C-4II cultures shows that such polarity can also be established autonomously by isolated epithelial cell populations. It is possible that the polarity of cytoplasmic organization in cultured cells may be determined, in the absence of heterologous tissues, by differential concentrations of the cells' own products at the cellular periphery. Extracellular materials, secreted randomly by initially nonpolar cells, could, upon adhesion, accumulate at high concentrations at the interface with the substratum, but not at the opposite, apical side which is exposed to the culture medium. A particular complement of contractile microfilaments in the basal cytoplasm of such adherent monolayer cultures might represent an intracellular tensile force which interacts with extracellular adhesive factors in the maintenance of colonial organization. Thus, the contraction of the cell sheets which followed their suspension in control medium could be the result of a sudden imbalance between persisting tensile forces in the basal cytoplasm and rapidly diminishing amounts of adjacent extracellular materials which, upon separation from the substratum, would be diffusing from the basal cell surface. Such a mechanism would imply that the convolution of the cell sheets was the result of a contraction or reorganization of microfilaments that already existed in the adherent, flat epithelia, where they were not identifiable electron microscopically, possibly due to more dispersed spacing. Alternatively, the changes in the basal environment upon suspension of the cells may have induced the formation of new microfilaments. The rapidity of the contraction, *i.e.* its onset within a few minutes upon suspension, points against its dependence on synthetic activity,

but would be compatible with the polymerization of preexisting subunits.

It is particularly interesting that, in the basal cytoplasm of suspended cells, the contraction and the formation of dense junctions were sensitive to cytochalasin B, while in the apical cytoplasm the integrity of the filament network and of its associated junctional complexes was not. If the function of the basal microfilaments in cells still attached to substrata should also be selectively inhibited by cytochalasin B, then it might be possible to determine what role, if any, these organelles might have in the adhesion of epithelial cells to underlying structures.

Preliminary observations indicate that the contraction of cultured epithelial monolayers, in response to suspension, is not limited to line C-4II. Similar although less drastic cytochalasin B-sensitive configurational changes have been observed in suspended cell sheets of the epithelial lines Hep 2, KB, and HeLa. However, the only cell line tested, so far, in which the response was as intense as in C-4II cultures, was line C-4I, a culture that, like C-4II, is of malignant squamous epithelial origin and has retained some properties of normal differentiated squamous cells (1).

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