EFFECT OF MICROTUBULE-DESTROYING DRUGS
ON THE SPREADING AND SHAPE OF CULTURED
EPITHELIAL CELLS

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SUMMARY
The role of microtubules in the spreading of cells from the liver-derived IAR2 rat cell line was
studied. Cells in control medium seeded on a flat isotropic glass surface rapidly spread to form
discoid shapes. Spreading in colcemid-containing medium was disorganized and delayed; partial
reversal of spreading was observed. Nevertheless, even in the presence of colcemid the cells finally
spread to discoid flattened shapes. IAR2 cells in medium without colcemid spread not to discoid but
to elongated shapes under three different sets of conditions: (1) when the cells were forced to spread
on narrow strips of adhesive glass surface between two non-adhesive lipid films; (2) when the cells
spread on the poorly adhesive surface of poly(HEMA)-covered glass; (3) when the cells spread on
the usual glass surfaces in medium containing cytochalasin D. Addition of colcemid to the media
reversed the polarized spreading under the first two conditions; colcemid did not reverse the
formation of the elongated cell shape acquired by the cells spreading in cytochalasin-containing
medium. Effects of microtubule-destroying drugs on the spreading of epithelial and fibroblast cells
are compared and discussed. It is suggested that microtubules are essential for the stabilization of
the spread state of those attached cytoplasmic processes and lamellae that do not have numerous and
stable cell-substratum contacts, e.g. the processes formed at the early stages of spreading or the
elongated processes of polarized cells. Possibly, microtubules stabilize the non- contracted state of
the actin cytoskeleton in these processes.

INTRODUCTION
The role of microtubules in the development and maintenance of the shape of tissue
cells at interphase was studied in detail in experiments with elongated polarized cells,
such as fibroblasts or neurons. It has been found that depolymerization of
microtubules by specific drugs prevents or reverses polarization of these cells. In
particular, fibroblasts become unable to achieve and to maintain elongated shapes;
differentiation of their edges into pseudopodially active and stable zones disappears
(Vasiliev et al. 1970; Vasiliev & Gelfand, 1976). Elongated cytoplasmic processes of
neuronal cells also disappear (Magendantz & Solomon, 1981). The role of the
microtubular system in the morphogenesis of the cells of the other main morphologi-
cal type, epitheliocytes, is much less clear. These cells spread to discoid shapes on the
usual isotropic culture substrata. Although the spread epithelial cells, like fibroblasts,
contain a well-developed system of microtubules occupying almost all their cytoplasm (Bershadsky et al. 1978), microtubule-destroying drugs have not been observed to cause any striking changes in their shape (Di Pasquale, 1975; Domnina, Pletyushkina, Vasiliev & Gelfand, 1977). The aim of experiments described in this paper was to examine the effects of a microtubule-destroying drug, colcemid, on the spreading of individual epithelial cells under various conditions. We have chosen IAR2 cells derived from rat liver for our experiments because these cells have a typical epithelial morphology that has been described in detail (Montesano, Saint Vincent, Drevon & Tomatis, 1975; Montesano et al. 1977; Bannikov, Saint Vincent & Montesano, 1980; Bannikov et al. 1982). In contrast to many other epithelial cells, which spread well only when they are in contact with one another (Middleton, 1976), single IAR2 cells spread easily on glass. Therefore, in experiments with this cell line it is easy to observe alterations in the morphology of individual cells in the course of spreading without the complicating effects of cell–cell contacts. We have examined the effect of colcemid on the final shape of spreading IAR2 cells and on the intermediate stages of their spreading. The effects on cells spreading on the usual substratum were compared with those exerted by colcemid under special conditions, in which epithelial cells were forced to undergo polarized spreading and to acquire not discoid but elongated shapes. Although, in agreement with previously published results, colcemid did not cause any striking alteration of well-spread discoid cells, this drug, under certain other conditions, was found to alter the morphology of epithelial cells as profoundly as that of fibroblasts; this happened, for instance, at the early stages of spreading on the usual substratum or in certain cases of forced polarized spreading. These data give reason to suggest that microtubules have an important common function in the spreading of cells of different tissue types; the possible nature of this function is discussed in the last part of this paper.

**Materials and Methods**

**Cell line and growth conditions**

The IAR2 epithelial cell line originally obtained from normal rat liver was used (see Introduction). The cells are not tumorigenic in syngeneic rats. The cell line was cultivated in William’s E medium (Flow Labs, Irvine, Scotland), supplemented with 10% foetal calf serum (Gibco-Biocult, Glasgow, Scotland) and 100 IU/ml monomycin. For scanning electron microscopy (SEM) and indirect immunofluorescence the cells were plated at an initial density of $2 \times 10^5$ to $5 \times 10^4$ cells/cm² on coverslips placed in Petri dishes and were cultured at 37°C in a humidified incubator supplied with 5% CO₂ in air. For phase-contrast or interference-reflection microscopy the cells were plated at an initial density of $2 \times 10^4$/cm² and living cells were viewed and photographed at 37°C in glass chambers as described by Vasiliev et al. (1970).

Colcemid (Serva, FRG) at 0.05–0.2 μg/ml or cytochalasin D (Serva, FRG) at 1–2 μg/ml were added to the culture medium at the time of cell plating, or at different intervals after cell seeding. Contact interaction of the cells with substrate surfaces of different adhesiveness was studied in experiments in which the coverslips were coated with a film of poly(2-hydroxyethyl methacrylate) (poly(HEMA); Hydron Lab., New Brunswick, N.J., U.S.A.). As shown by Folkman & Moscona (1978) the adhesiveness of plastic tissue culture can be reduced in a graded manner by applying increasing concentrations of poly(HEMA). We used serial dilutions (1/2000, 1/1000, 1/600 or 1/250) of the ethanol/poly(HEMA) solution for coating the coverslips. For examination of cell
Microtubule-destroying drug, effect on epithelium

orientation and cell elongation the cultures were plated on coverslips coated with a non-adhesive thin film of lecithin, prepared from lecithin solution (2 mg/ml), which was a kind gift from Dr L. B. Margolis (this laboratory). Narrow linear scratches on the glass surface were made in the lecithin film with a sharp needle as described by Ivanova & Margolis (1973).

Scanning electron microscopy
The cells were fixed in sodium cacodylate-buffered isotonic 2% glutaraldehyde (pH 7.2). After washing, the specimens were dehydrated in increasing concentrations of the water/acetone solution and dried in a critical-point drier (Balzers Union, Liechtenstein). The cells were coated with gold/palladium in a cold sputter (Polaron Equipment Ltd, England) and examined in a Cambridge Stereoscan S4 microscope.

Antibodies and immunofluorescence microscopy
Monospecific rabbit antibodies against chicken gizzard actin and bovine brain tubulin have been characterized elsewhere (Bershadsky, Gelfand, Svitkina & Tint, 1980). Monoclonal antibodies against vimentin, clone NT30, were kindly provided by Dr A. A. Neifakh and Dr I. S. Tint (All-Union Cancer Research Center, Moscow). For indirect immunofluorescence microscopy, cultures were washed thoroughly in phosphate-buffed saline (PBS) and fixed with 4% formaldehyde in PBS. Before fixation the cells were extracted for 3 min with 1% Triton X-100 in imidazole buffer, as described earlier (Bershadsky et al. 1980). Observations were made with a Zeiss Photomicroscope III (Carl Zeiss, FRG) equipped with epifluorescent illumination and a Planapo 40× oil-immersion objective.

RESULTS

Spreading in control medium
Control cells spreading on glass (Fig. 1) formed the first narrow outgrowths (filopodia), seen by SEM and by phase-contrast, at 5–7 min after the first contact with the substratum. Soon after that, at 7–10 min, numerous flattened lamellipodia (ruffles) were formed around the cell body, which at this stage was still almost spherical. Formation and gradual expansion of the ring of lamellar cytoplasm from the substratum-attached ruffles took place during the next 20–40 min. Ruffling continued at the peripheral edges of this ring; circular ruffles moving centripetally across the upper surface of the lamellar cytoplasm were often seen during this period. Simultaneously with the widening of the ring of lamellar cytoplasm, gradual flattening of the central cell body took place. At 60 min most cells had already acquired the final discoid shape with a flat upper surface covered only with short microvilli. Ruffles formed at the edges of these discoid cells were smaller than those seen at earlier stages of spreading; 24 h later the cells retained the same shape, except that the ruffling had stopped almost completely and the outer edges had acquired smooth circular shapes. After divisions the daughter cells remained in close contact with one another so that coherent small cell groups were formed. Any significant translocation of single cells or of cell groups on the substratum was not observed. By IRM, first small dot-like focal contacts were seen at the periphery of the cells at 10–30 min after seeding; the fully spread discoid cells had a regular ring of black or dark-grey contacts at the
Fig. 1
periphery; some contacts forming this ring were dot-like, while others were linear and oriented tangentially to the edge (Fig. 2). Circular bundles of microfilaments were revealed by anti-actin antibody in the lamellar cytoplasm of discoid cells; straight bundles crossing the internal parts of the cytoplasm of these cells were seen only rarely. In the cell groups peripheral marginal belts of microfilament bundles were better developed near contact-free lateral edges than near internal edges (Fig. 3). Microtubules radiated from the perinuclear zones of discoid cells into their lamellar cytoplasm and often formed arcs there (Fig. 4). Peripheral parts of the microtubules often formed marginal bundles almost parallel to the cell edge. Intermediate filaments revealed by antibody against vimentin formed a loose network in the cytoplasm (Fig. 5A). As shown in our laboratory, the IAR2 line, in contrast to many other epithelial cells lines, did not have any intermediate filaments containing pre-keratin (Troyanovsky, personal communication).

The cells spreading on narrow strips of glass between non-adhesive phospholipid films, at 24 h after seeding acquired highly elongated shapes with small lamellae at one or both ends and with badly spread fusiform central bodies (Fig. 6A,B). Ruffling was seen at the edges of the lamellae of these cells; their lateral edges were inactive. By IRM diffuse zones of close contacts were seen at various sites on the ventral surfaces of these cells, with occasional small black zones near the edges.

The cells seeded on poly(HEMA) diluted 1:2000 or 1:1000, 24 h later had the same shapes as the cells spread on glass (Fig. 7A). The degree of spreading at 24 h decreased when the cells were seeded on poly(HEMA) diluted 1:600, and especially on poly(HEMA) diluted 1:250. At 24 h most cells on this last substratum had poorly spread dome-like or elongated fusiform shapes (Fig. 7B).

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**Fig. 1.** Consecutive phase-contrast micrographs of an IAR2 cell spreading on glass in control medium. Time after seeding: A, 12 min; B, 23 min; C, 25 min; D, 28 min; E, 41 min; F, 1 h 15 min. Bars, 20 μm.

**Fig. 2.** The ring of contacts of a spread discoid cell, 24 h after seeding in control medium: A, phase-contrast; B, interference-reflection microscopy. Bars, 20 μm.

**Fig. 3.** Peripheral microfilament bundles in a group of cells spread in control medium 24 h after seeding. Indirect immunofluorescence using anti-actin antibody. Bar, 30 μm.

**Fig. 4.** Microtubules in the control cell, 24 h after seeding. Indirect fluorescence using anti-tubulin antibody. Bar, 20 μm.

**Fig. 5.** Indirect immunofluorescence using antibody against vimentin; 24 h after seeding: A. Cell in control medium. Network of intermediate filaments in the cytoplasm; B, cell incubated with 0·1 μg/ml colcemid for 24 h. A ring of collapsed filaments can be seen around the nucleus. Bars, 10 μm.

**Fig. 6.** A,B. Elongated IAR2 cells spread on narrow strips of glass surrounded by non-adhesive phospholipid film. Phase-contrast; 24 h after seeding. Bars, 30 μm.

**Fig. 7.** Cells spread on poly(HEMA)-covered substrate, 24 h after seeding. Scanning electron microscopy: A. Well-spread discoid cell in poly(HEMA) diluted 1:2000; B, poorly spread elongated cell on poly(HEMA) diluted 1:250. Bars, 15 μm.

**Fig. 8.** Cells in control medium (A) and in colcemid-containing medium (0·1 μg/ml) (B), 1 h after seeding. Scanning electron microscopy. Notice numerous blebs at the surface of the colcemid-treated cell. Bars: A, 15 μm; B, 5 μm.
Figs 2–5. For legend see p. 271
Microtubule-destroying drug, effect on epithelium

Figs 6–7, 9. For legend see p. 271
Fig. 8
Effects of colcemid

The cells spreading on the usual glass substrate in colcemid-containing medium (Figs 8, 9a) began to form numerous blebs at their periphery at 5–60 min after contact with the substrate. Most blebs were 5–10 μm in diameter; large elongated balloon-like processes (lobopodia) were also formed. The blebs appeared and disappeared very quickly; some of them existed for only a few minutes. Besides the blebs, attached lamellipodia were seen at the cell periphery at 1–1.5 h; formation of the narrow circular ring of lamellar cytoplasm from these lamellipodia took place at 1.5–2.0 h after seeding, that is, much later than in control cultures. At 2–4 h large blebs were often seen at the upper surfaces of lamellar rings. In contrast to the controls, the spreading of these cells often underwent partial reversal: some parts of the lamellar ring detached themselves from the substrate and contracted. In spite of these reversals the average degree of cell spreading gradually increased. At 6–8 h after seeding most cells had acquired relatively well-spread discoid shapes; further reversal of spreading...
Figs 10-12. For legend see p. 275
Figs 13–18. For legend see p. 275
was not observed. Discoid cells seen at 16–24 h in colcemid-containing medium often had certain morphological differences from the discoid cells seen at the same times in control cultures: (1) the contours of their outer edges usually were not smooth but indented; they had numerous short flat extensions up to 5 µm in length and 1–5 µm in width. Occasionally blebs and lobopodia were also seen at the edge; (2) the lamellar cytoplasm was somewhat smaller in size; it was thicker near the edges than in more central parts; (3) the central part of the cell body was less flattened and more elevated than in controls.

When cells spread in control medium for 24 h were transferred into colcemid-containing medium, during the next 1–2 h they became indistinguishable from cells kept continuously in this last medium: their contours became less smooth, the area of lamellae became somewhat decreased and their central part became thicker (Fig. 10A,B). Removal of colcemid from the medium lead to restoration of normal morphology. By IRM, discoid cells in colcemid-containing medium, like control cells, had a ring of contacts but the width of this ring was wider and more variable (Fig. 11). Increased variability in the width of the marginal ring of actin was also observed. Incubation of colcemid-treated cells with anti-tubulin antibodies did not reveal any microtubules in the cytoplasm. Most intermediate filaments revealed by antivimentin antibody collected around the nucleus forming brightly fluorescent rings (Fig. 5A).

Effects of vinblastin (0.05–0.2 µg/ml) on the course of spreading were similar to those of colcemid. The cells seeded in colcemid-containing medium on narrow strips of glass between two lipid films did not spread to form elongated shapes; at 24 h these cells still had rounded, almost unspread, shapes with numerous blebs at the periphery (Fig. 12). When cells spread in control medium on narrow strips were transferred into the colcemid-containing medium, rapid detachment of their elongated processes took place; and the cells also acquired rounded shapes. The elongated shapes were restored after transfer into drug-free medium. The cells on the substrate covered with 1/250 poly(HEMA) also did not spread to elongated shapes in colcemid-containing medium. At 24 h most cells remained almost spherical and could be easily washed off the substrate.

**Effects of cytochalasin D**

Several types of experiments with cytochalasin D were performed.

(1) Cells were spread for 24 h on glass in control medium and transferred into medium containing cytochalasin D (CD-medium). During the next hour their discoid lamellae contracted leaving a network of branching cytoplasmic strands on the sub-stratum; this network surrounded an elevated central cell body (Fig. 13).

(2) Cells were spread for 24 h on narrow strips of glass in control medium and then transferred into CD-medium. During the next 1–2 h two peripheral lamellae of these elongated cells were transformed into a tree-like system of thin cylindrical processes (Fig. 14).

(3) Suspended cells were seeded on the usual glass substratum in CD-medium. During the first 2–4 h of spreading these cells formed several narrow elongated
processes at opposite edges of the cell body. Later these processes increased in length so that by 24–48 h the cells had acquired elongated dipolar shapes (Fig. 15). Flattened lamellae were not formed at the edges of these cells; by IRM, numerous dot-like dark-grey and black contacts were seen at the lower surfaces of these cells. Addition of colcemid (0.1 µg/ml) to CD-medium 24 h after seeding did not lead to the disappearance of elongated processes 2–4 h later, although the thickness of these processes became more variable (Fig. 16). After 24 h of incubation with colcemid, the average thickness of these processes had decreased considerably (Fig. 17). Transfer of the elongated cells from CD-medium into control medium was followed by gradual flattening and widening of the cellular processes until, 24 h after the transfer, the cells acquired ellipsoidal shapes (Fig. 18); at 48 h the usual discoid shapes were restored.

(4) Suspended cells were seeded on the usual substratum in medium containing both CD (1–2 µg/ml) and colcemid (0.1–0.2 µg/ml). These cells remained non-spread for the next 24 h and formed only small rudimentary pseudopodia at various parts of the cell perimeter.

**DISCUSSION**

*Isotropic and non-isotropic spreading of epithelial cells*

IAR2 cells, like other epithelial cells, spread isotropically to form discoid shapes on the usual substrata. Unlike fibroblasts, these cells do not undergo spontaneous polarization. However, we have found that IAR2 cells can spread anisotropically to form polarized shapes in three sets of special conditions: on narrow strips of adhesive substrate, on poly(HEMA) substrate and on the usual substrate in cytochalasin-containing medium.

The mechanism of polarization is most obvious in the first case: cells spreading on narrow strips are able to attach their pseudopodia only along the strip and thus undergo forced polarization.

When cells spread on a poorly adhesive substrate, such as poly(HEMA), then simultaneous attachment of pseudopodia extending in all directions becomes improbable; but the cells can still acquire elongated shapes, which requires successful attachment of pseudopodia only in two opposite directions. Studies of the effects of cytochalasins on the spreading of fibroblasts have shown that these drugs decrease the number of sites on the cell edge from which pseudopodia are extended as well as the width of the attached pseudopodia (Bliokh et al. 1980; Domnina et al. 1982).

High efficiency of pseudopodial attachment and rapid widening of the lamellae are essential for isotropic spreading. As suggested earlier (Vasiliev, 1982), other conditions being equal, all the factors decreasing the efficiency of extension and attachment of pseudopodia should favour the transition from isotropic to anisotropic spreading. Results of experiments with poly(HEMA) and with cytochalasin are in good agreement with this suggestion.

It is interesting that neoplastically transformed IAR cells, in contrast to non-transformed parents, often acquire elongated polarized shapes even on the usual
culture substratum. This elongation is probably a manifestation of the decreased efficiency of their spreading (Bannikov et al. 1982).

Effects of colcemid on isotropic and anisotropic spreading of IAR2 cells

All the effects of colcemid on IAR2 cells observed in our experiments were induced by relatively small concentrations of the drug, which resulted in complete disappearance of cytoplasmic microtubules but were not visibly toxic. These effects were completely reversed by the removal of the drug from the medium. Another inhibitor of the polymerization of microtubules, vinblastin, had essentially the same effects as colcemid. These facts give us reason to think that the effects of colcemid on spreading are specific, that is, associated with depolymerization of microtubules.

We have found that colcemid completely prevents polarization of IAR2 cells on special substrates, such as poly(HEMA) and adhesive strips. This drug also disorganizes the early stages of isotropic spreading on the usual substrate. Once reached, the well-spread discoid shapes of IAR2 cells become relatively resistant to colcemid. These effects are essentially similar to the effects of microtubule-destroying drugs on fibroblasts described by Ivanova, Margolis, Vasiliev & Gelfand (1976).

The effects of colcemid on fibroblast cultures are more obvious, because in this case cells spread on the usual substrates acquire polarized shapes that are highly sensitive to colcemid. Therefore, the differences between the control and colcemid-treated cultures are easy to see at any time after the end of spreading. In contrast, morphological differences between drug-treated and control epithelial cultures become less striking at the final stages of spreading.

To explain the observed effects of colcemid and similar drugs we suggest that microtubules counteract the contractility of the actin cortex. Owing to this contractility pseudopods and lamellae that had extended and attached during spreading tend to retract and to detach themselves from the substrate (see review by Harris, 1982). Possibly, microtubules, when present, diminish these effects of contractility and therefore stabilize the shape of attached processes. This suggestion is supported by the experiments of Magendantz & Solomon (1981).

In these experiments drug-induced depolymerization of microtubules in cultured neuroblastoma cells lead to contraction and disappearance of long axon-like processes; this contraction was prevented by simultaneous treatment of these cells with cytochalasin. The results of our experiments with IAR2 cells were essentially similar: the only variant of polarization that was not reversed rapidly by colcemid was that obtained in the cytochalasin-containing medium, that is, under the conditions in which the contractility of actin cortex was impaired.

The different sensitivity of various stages and types of spreading to colcemid can also be explained by the opposite effects of microfilaments and microtubules on the stability of attached pseudopodia. Obviously, pseudopodia can retract more easily at the early stages of spreading when cell–substrate attachments are not yet numerous; therefore, prevention of excessive contraction of the microtubules is essential for rapid progress of spreading through these stages. Microtubules are also essential under conditions in which a group of contacts of the attached lamellae is separated from
other attachments by an elongated stretched process, that is, in polarized cells. In contrast, microtubules are less important for the prevention of retraction of lamellae of non-polarized discoid cells, which have numerous attachments with the substrate along the whole cell perimeter. It is significant that depolymerization of microtubules causes small retraction of lamellae even in these discoid cells.

We do not know how microtubules prevent retraction of extended pseudopodia. They can act mechanically as stiff rods counteracting the contractility of the actin cortex. More complex interactions of microtubules with the actin cytoskeleton are also possible. For instance, microtubules can direct the transport of non-polymerized actin towards the sites of polymerization of new microfilaments, especially to the active edges. It has been suggested (Dunn, 1980) that an ‘actin flow’ is essential for pseudopodial extension. It is also well known that destruction of microtubules disorganizes many types of intracellular movements (see review by Schliwa, 1984). All these suggestions require further experimental tests.

Destruction of microtubules in IAR2 cells and in many other cell types (see review by Lazarides, 1980) is accompanied by the collapse of vimentin-containing intermediate filaments. Are the effects of colcemid on spreading of these cells mediated by alterations in the intermediate filaments? Selective collapse of intermediate filaments induced by the injection of specific antibody did not lead to any obvious alteration in cell shape (Klymkowsky, Miller & Lane, 1983). Recently, we (Karavanova, Troyanovsky & Vasiliev, unpublished) found that colcemid reverses polarization of elongated mouse hepatoma cells containing cytokeratin intermediate filaments; incubation of these cells with colcemid destroyed their microtubules but did not lead to the collapse of intermediate filaments. Therefore, it is unlikely that intermediate filaments play an essential role in the effects of destruction of the microtubular system on the shape of epithelial cells.

References


(Received 3 July 1984–Accepted 16 October 1984)