Length control is determined by the pattern of cytoskeleton

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Summary

In our previous experiments with linear strips of adhesive substrate, we found that elongated cultured fibroblasts preserve their length regardless of cell width and the number of cytoplasmic processes. This constancy of length was called 'length control'. In contrast to fibroblasts, single cultured epitheliocytes have nearly discoid shape on the plane substrata and have no length-controlling mechanism: their length on the narrow strips of adhesive substrate increased significantly in comparison with the diameter on the plane substrate. These results suggested that control of length is cell specific. An alternative suggestion is that length control is associated not with the cell type but with the cell cytoskeletal pattern (namely, with epithelioid circular actin bundles or straight actin bundles). Experiments described in this paper were made to choose between these two suggestions. Mouse embryo fibroblasts spreading on the planar substrate first acquire discoid epithelioid shape with a circular actin bundle. Only later did they acquire a polarized shape with straight actin bundles. Polarized, fully spread fibroblasts temporarily acquire discoid epithelioid shape when treated with the Taxol, disorganizing microtubules. However, epithelial discoid cells can be transformed into elongated fibroblast-like cells by scatter factor (HGF/SF; a cytokine) and by agents inhibiting Rho kinase. These reversible transitions from fibroblastic to epithelioid shape and vice versa were accompanied by a corresponding disappearance and appearance of length control. Fibroblasts with stress fibers destroyed by the Rho-kinase inhibitor Y27632 became considerable longer on the adhesive strips than on the plane while retaining a near-polarized shape. Thus, length control is typical not of the cell origin but of the cell phenotype (i.e. for polarized cells with microtubules and intact actin cytoskeleton).

Key words: Microtubules, Microfilaments, Y 27632, Morphometry, Cell shape, Micropatterned adhesiveness

Introduction

In experiments with linear strips of adhesive substrate, it has been found that elongated cultured fibroblasts preserve their length regardless of cell width and number of cytoplasmic processes. In contrast to fibroblasts, single cultured epitheliocytes have a nearly discoidal shape on the plane substratum and have no length-controlling mechanism: their length on the narrow strips of adhesive substrate increased significantly in comparison with the diameter on the plane substrate (Levina et al., 2001). Comparison of three types of fibroblasts (mouse embryo fibroblasts plus human fibroblastic lines AGO 1523 and M19) with three epitheliocyte cell lines (rat IAR2, canine MDCK and bovine FBT) confirmed that only fibroblasts, not epitheliocytes, maintain similar lengths on the conventional substrates and on the linear adhesive strips. These results suggest that control of length is cell-type specific.

However, length control might be associated not with the cell type but with the cell's cytoskeletal pattern. It is well known that fibroblasts, when fully spread, usually have straight bundles of actin microfilaments (stress fibers) running through the cell body approximately parallel to the body axis and microtubules running from perinuclear part toward the cell periphery. However, in the course of spreading on the plane substrate, fibroblasts usually pass through a transitory phase when they have discoid shape and a circular microfilament bundle (the so-called radial stage) (Vasiliev, 1985). Polarized, fully spread fibroblasts can temporarily acquire discoid, epithelioid shape when treated by drugs that disorganize microtubules such as Taxol® (Pletjushkina et al., 1994) or a combination of drugs inhibiting Rho kinase and drugs that depolymerize microtubules (Omelchenko et al., 2002). However, epithelial discoid cells can be transformed into elongated fibroblast-like cells by cytokines such as scatter factor [cytokine hepatocyte growth factor/scatter factor (HGF/SF)] (Stoker et al., 1987). In the experiments described in this paper, the state of length control was revealed by comparison of morphometric parameters of the cells cultivated on the planar substrate (glass) and on the specially prepared linear adhesive strips of the same substrate. These experiments showed that these reversible transitions from epithelioid to fibroblastic shape and vice versa are accompanied by a corresponding appearance and disappearance of length control. At the same time, fibroblasts with stress fibers destroyed by Rho-kinase inhibitor lost length control while retaining a near-polarized shape. Thus, length control is a characteristic not of the cell origin but of the cell phenotype (i.e. of polarized cells with stress fibers).

Materials and Methods

Cell cultures

The cells of two different morphological types were used in the

experiments: fibroblasts (mouse embryo fibroblasts) and two variants of epitheliocytes [rat liver epithelial cells (IAR-2) and the canine kidney MDCK line]. Cultures of mouse fibroblasts were prepared by trypsinization of day 14-15 embryos. IAR-2 epithelial cells were derived from rat liver (Montesano et al., 1975). MDCK epitheliocytes were derived from canine kidney (Cereijido et al., 1978).

The cells were grown in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% fetal calf serum (Gibco Biocult, UK) at 37°C in humidified incubator supplied with 5% CO_2 in air. The cells were plated at an initial density of 100 cells mm^{-2} on control substrates or 60 cells mm^{-2} on the special substrates with narrow linear adhesive strips (see below), and placed in 30-mm tissue culture dishes. Owing to the low initial cell density, most cells (about 70%) on both substrates remained single after 24 hours; that is, they had no visible cell-cell contacts.

Taxol (Sigma; 10 μg ml^{-1}), Y27632 (Calbiochem, USA; 15 μM) or scatter factor (Sigma, USA; 10 nM) were added to the culture medium 20 hours after cell plating. The cells were incubated overnight with Taxol and Y27632 and for 22 hours with scatter factor before examination.

Substrates

We used glass coverslips (Chance Propper, Smethwich, UK) as the normal plane substrates with isotropic adhesive surfaces. Substrates with narrow (15±3 μm) linear strips of adhesive surface were prepared as previously described (Levina et al., 2001).

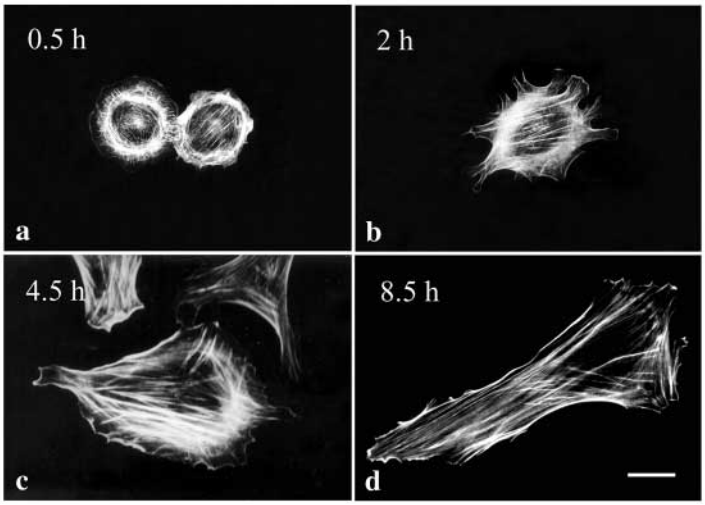
Differential interference contrast microscopy

The live 24-hour-old cultures were examined by video-enhanced microscopy using a Zeiss Axiophot microscope equipped with differential interference contrast (DIC) optic system with a 40×0.7 Pl Fluotar objective and Hamamatsu Newvicon videocamera (Hamamatsu, Japan). To record images, we used a video tape recorder (SVT-S3050P, Sony, Japan).

Morphometric analysis of cell shape.

The outlines of DIC images of single cells (i.e. of the cells without any cell-cell contacts) were used for morphometric analysis (Levina et al., 2001). Shape characteristics such as maximal cell length, cell area and dispersion and elongation indices were calculated.

Cell length was defined as the length of direct line between the furthest two points on a cell outline. Average values of length of groups of cells were calculated. They are designated in the text as 'average length' of cell population.



Dispersion and elongation indices were determined and calculated as described by Dunn and Brown (Dunn and Brown, 1986). These parameters detect fundamental transformations of cell shape. Elongation and dispersion indices describe two different aspects of how a shape differs from a circle. Both measure how much the total 'mass' of the shape extends away from its 'center of gravity' but elongation describes how much this extended mass can be reduced by compressing the shape along its long axis and dispersion describes how much extended mass remains. Thus, elongation describes how elliptical a cell is, whereas dispersion describes how irregular a cell's edges are.

For the calculation, no fewer than 50 cell outlines were used for each case. Data are expressed as the mean±s.e.m. Comparisons between two groups were made using Student's *t* test. *P* values <0.05 were considered to be significant.

Fluorescence microscopy

After 24 hours of culture, cells were washed with PBS, fixed in 3% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1% Triton X-100 for 1 minute at room temperature. For tubulin staining, the cells were fixed with methanol at –20°C for 10 minutes. F-actin was stained with TRITC-conjugated phalloidin (Sigma Chemical, St Louis, USA). Tubulin was stained with anti-α-tubulin mouse monoclonal IgG1 (clone DM1-A, Sigma, USA). As secondary antibodies, we used Oregon-Green-488-conjugated goat anti-mouse IgG (Molecular Probes, USA). After several rinsings in PBS, preparations were mounted in buffered polyvinyl alcohol (Lennett, 1978). Fluorescence microscopy was performed using an Aristoplan microscope (Leitz, Germany) equipped with epifluorescence illumination and a 50× 1.0 Pl Fluotar water-immersion objective.

Results

Length control in the course of spreading of fibroblasts

In the course of spreading on planar isomorphic substrate such as glass or tissue culture plastic cultured primary fibroblasts pass through several morphological stages. The first is the discoid or radial stage, when spheroidal cells parachuting onto the substrate from suspension extend and attach lamellipodia from the lower part of their bodies in all radial directions; simultaneously the central body gradually flattens so that the cell eventually acquires a discoidal shape. A circular bundle of actin microfilaments is characteristic of this stage (Fig. 1a-c). The second is the polarization stage, when the formation and attachment of lamellipodia continues at some zones of the cell edges but stops at others, so that the cell gradually acquires a polygonal shape and then the characteristic elongated 'fibroblast-like' shape (elongated with one or several cytoplasmic processes and lamellipodium-forming zones) (Fig. 1d). During polarization, the circular actin bundle disappears and straight bundles (stress fibers) are formed that cross the cell body and have focal adhesions at the ends. Microtubules radiate from perinuclear zone into peripheral cell regions.

Morphometric indices such as elongation and dispersion reflect these characteristic shape changes (Table 1). During the first discoid stage (up to 3 hours), dispersion indices are near zero and elongation indices are also lower than 0.5.

Fig. 1. Shape and actin cytoskeleton organization of mouse embryo fibroblasts spreading on control substrate. Fluorescent microscopy after staining for actin. Scale bar, 20 μm.

Table 1. Morphometric parameters of fibroblasts and epitheliocytes*

	Control substrate			Substrate with adhesive strips		
	Area (μm^2)	Dispersion index	Elongation index	Area (μm^2)	Dispersion index	Elongation index
Mouse embryo fibroblasts						
Control	5264.0±238.5	0.57±0.03	1.64±0.05	1522.0±83.8	0.30±0.02	3.98±0.05
Taxol	3492.4±121.1	0.03±0.004	0.39±0.03	1011.2±54.5	0.10±0.01	3.74±0.09
Control	5509.2±325.2	0.49±0.05	1.11±0.08	1422.4±63.8	0.18±0.01	4.20±0.07
Y27632	4864.9±328.4	1.47±0.13	1.41±0.12	2762.9±189.3	0.51±0.04	4.88±0.12
IAR2 epitheliocytes						
Control	2278.7±82.4	0.048±0.005	0.71±0.04	1147.8±50.7	0.087±0.012	3.30±0.09
Y27632	3285.2±151.4	0.87±0.08	0.84±0.08	1616.4±75.6	0.16±0.01	4.50±0.08
MDCK epitheliocytes						
Control	720.5±72.9	0.025±0.005	0.34±0.04	609.8±33.6	0.12±0.02	3.01±0.14
SF	795.4±35.0	0.68±0.08	2.09±0.14	526.7±24.6	0.13±0.01	2.88±0.10

*Mean±s.e.m. are given. No fewer than 50 cell outlines from three experiments were used for the calculations in each group.

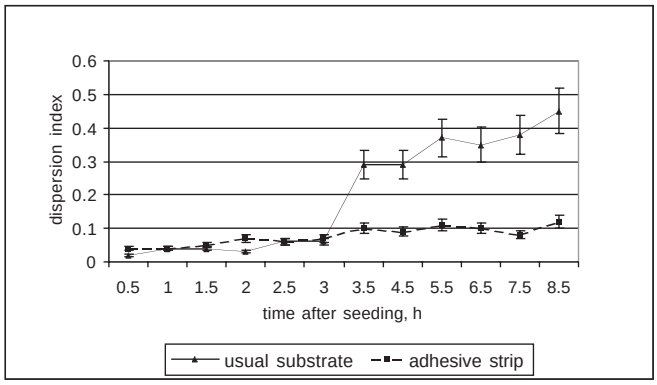


Fig. 2. Dispersion indices of mouse embryo fibroblasts on usual substrate and adhesive strips during spreading (means±s.e.m.).

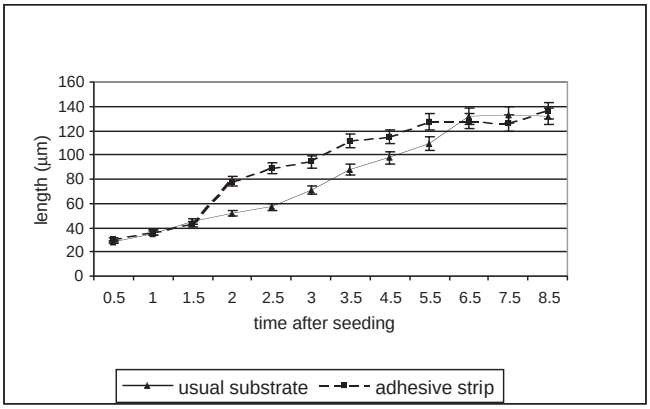


Fig. 3. Length of mouse embryo fibroblasts on usual substrate and adhesive strips during spreading (means±s.e.m.).

Later, progressive polarization is manifested by the continuous growth of both indices. The two-stage character of spreading on the adhesive strips is not so obvious visually and morphometrically: these cells have no lateral processes and their dispersion indices accordingly remain low during the whole spreading period (Fig. 2).

The comparison of maximal length on the plane and on the adhesive strips gave the most interesting results. From 2 hours to 5.5 hours after seeding, length on the adhesive strips remained significantly higher that on the plane, whereas, at 6 hours and later, these lengths became similar (Fig. 3). Analysing the dynamics of disappearance of circular actin bundles, we concluded that the first time interval corresponds approximately to the discoid stage of spreading on the plane and the second to the polarization stage (Fig. 4). We concluded that the average lengths on the plane and on the adhesive strips are equalized at the polarization stage.

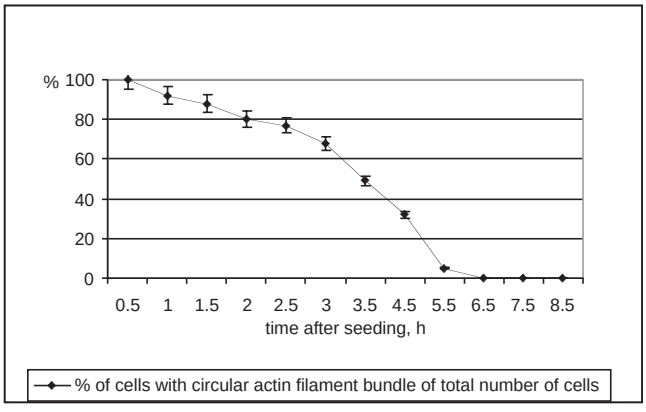


Fig. 4. Proportion of cells with circular bundles of actin filaments during spreading of mouse embryo fibroblasts (means±s.e.m.).

Microtubule-specific drugs

Mouse embryo fibroblasts on standard glass substrate have polygonal bodies, often with two or three cytoplasmic processes (Fig. 5a); the same fibroblasts on a adhesive strip are elongated and oriented along the adhesive strip, actin microfilament bundles are oriented along the adhesive strip (Fig. 5b). Taxol abolishes polarization of fibroblasts and most Taxol-treated cells on the plane have regular discoidal shapes with circular actin bundles at the periphery (Fig. 5c) and short microtubule

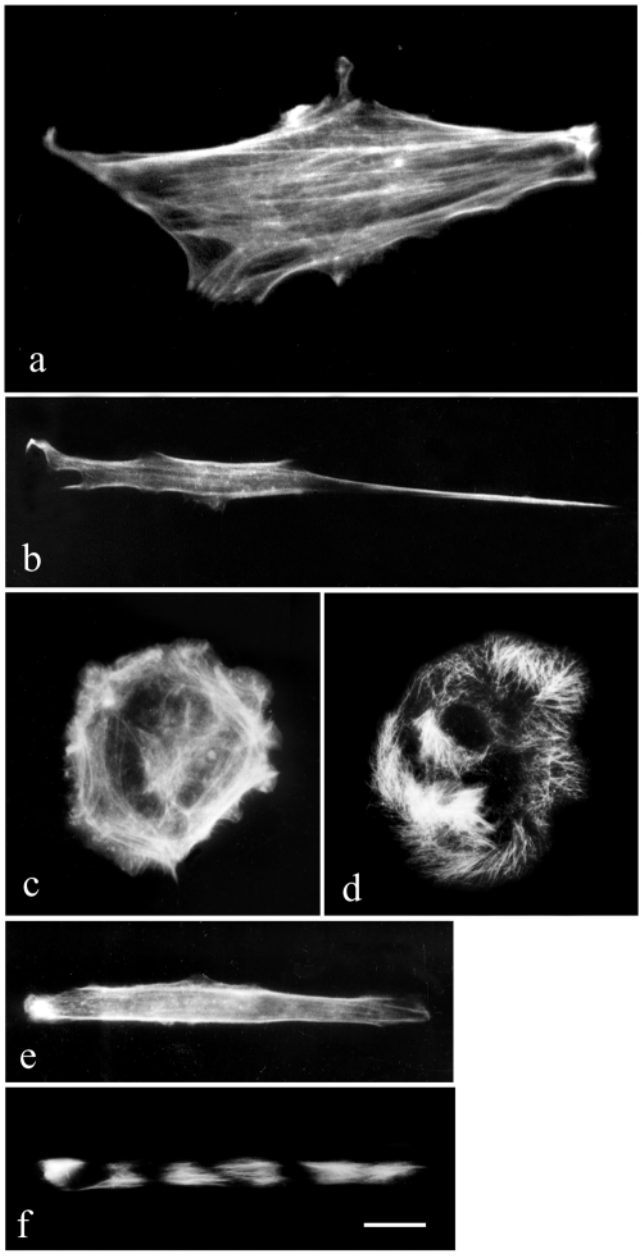


Fig. 5. Morphology and cytoskeleton organization of mouse embryo fibroblasts treated with Taxol on the control substrate or on the substrate with narrow adhesive strips. (a,b) Control cells. (c-f) Taxol-treated cells. (a,c,d) Cells on the control substrate. (b,e,f) Cells on the substrate with narrow adhesive strips. Fluorescent microscopy after staining for actin (a,b,c,e) and tubulin (d,f). Scale bar, 20 μm.

fragments scattered in the cytoplasm (Fig. 5d), and dispersion indices that are significantly lower (Table 1). On the adhesive strips, Taxol-treated cells had elongated shapes (Fig. 5e,f) with lengths significantly greater than on the plane (Fig. 6). Thus, Taxol-induced loss of polarization on the plane was accompanied by loss of length control on the adhesive strips.

Effects of scatter factor on epitheliocytes

Scatter factor (HGF/SF) is a protein that specifically induces

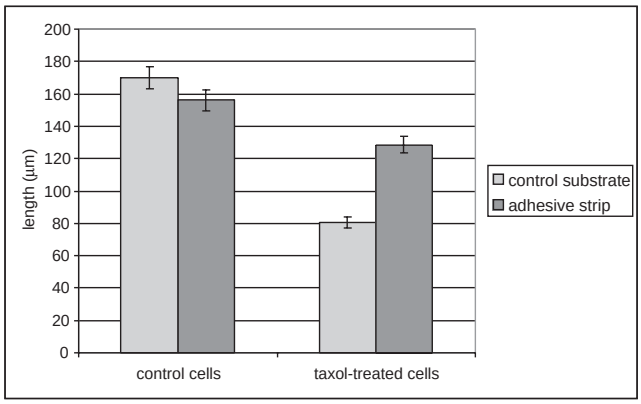


Fig. 6. Length of mouse embryo fibroblasts on control substrate and adhesive strips after Taxol treatment (means±s.e.m.). The difference between length on strips and on plane is highly significant for Taxol-treated culture but not for control culture.

the transformation of epitheliocytes into elongated fibroblast-like cells, the so called epithelio-mesenchymal transformation. The IAR2 rat epitheliocytes used in our experiments are insensitive to HGF/SF so, to test the effects of epithelio-mesenhymal transformation, we used another epithelial line, canine MDCK cells (clone 20), which is highly sensitive to HGF/SF. MDCK epitheliocytes have circular actin bundles at their periphery (Fig. 7a); on the adhesive strip, epitheliocytes have an ellipsoidal shape (Fig. 7b). After scatter-factor-induced polarization of MDCK epitheliocytes (Fig. 7c), the microfilament bundles disappeared (Fig. 7c,d). As shown in Table 1, incubation with scatter factor changed the morphometric indices of these cells to those typical of fibroblasts and simultaneously restored length control: significant differences between average lengths on the plane and on the adhesive strips disappeared after incubation with HGF/SF (Fig. 8, Table 1).

Effects of RHO-kinase inhibitor Y27632 on fibroblasts and epitheliocytes

IAR2 epitheliocytes have circular actin bundles at their periphery (Fig. 9a); on the adhesive strip, epitheliocytes acquire an ellipsoidal shape with circular actin bundles arranged along the whole cell periphery (Fig. 9c). As described earlier, Y27632 is a selective inhibitor of Rho kinase that enhances process formation by fibroblasts on the plane and induces process formation by discoid IAR2 epitheliocytes (Fig. 9b, Table 1). In both types of cell, microfilament bundles disappeared; the circular actin bundles of epitheliocytes are not present after incubation with Y27632 (Fig. 9b,d). In fibroblasts, Y27632 did not significantly affect average cell length on the plane but strongly increased it on the adhesive strips (Fig. 10). We have also found (S. A. Minina and J.M.V., unpublished) that another inhibitor of actin-myosin contractility, HA-1077, considerably increases the average length of fibroblasts on the plane.

In IAR2 epitheliocytes, Y27632 significantly increased length on the plane and on the adhesive strips compared with corresponding untreated control cells (Fig. 11). In both control

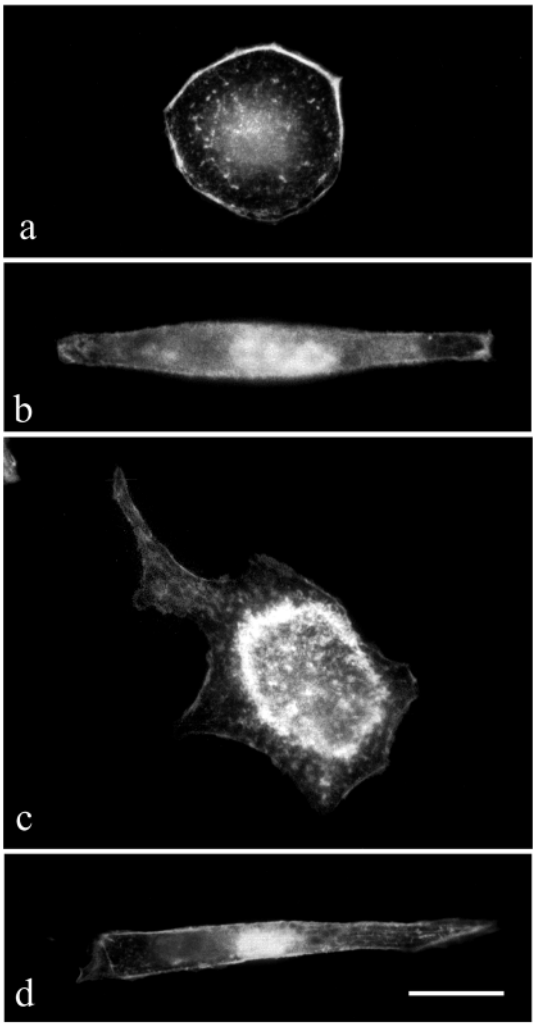


Fig. 7. Morphology and actin cytoskeleton organization of MDCK epitheliocytes treated with scatter factor on the control substrate or substrate with narrow adhesive strips. (a,b) Control cells. (c,d) Scatter-factor-treated cells. (a,c) Cells on the control substrate. (b,d) Cells on the substrate with narrow adhesive strips. Fluorescent microscopy after staining for actin. Scale bar, 20 μm.

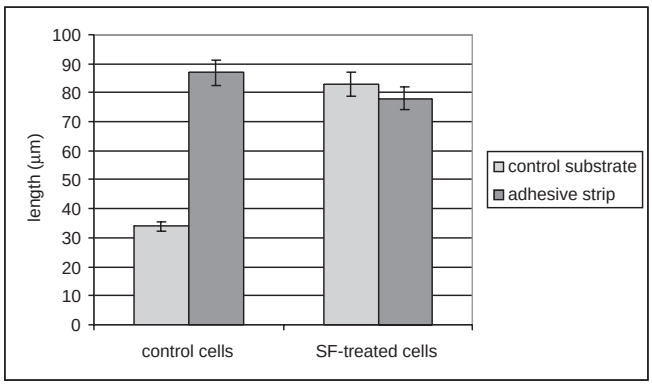


Fig. 8. Length of MDCK epitheliocytes on control substrate and adhesive strips after scatter-factor treatment (means±s.e.m.). The difference between length on strips and plane is highly significant for control culture but not for scatter-factor-treated culture.

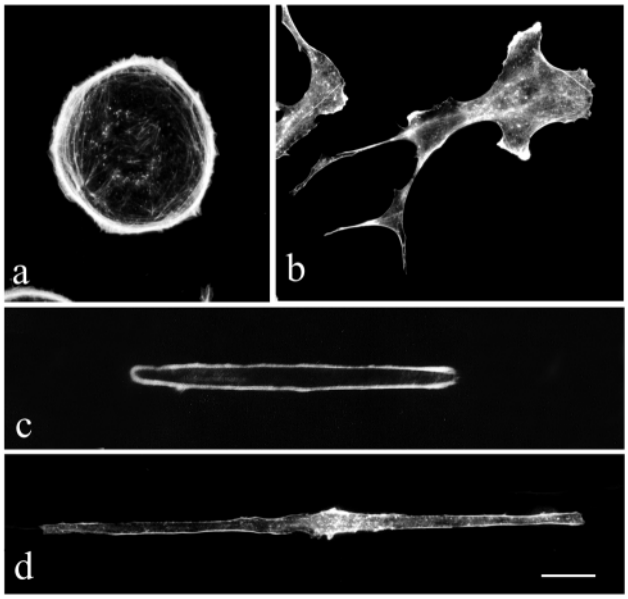


Fig. 9. Morphology and actin cytoskeleton organization of IAR2 epitheliocytes treated with Y27632 on the control substrate or on the substrate with narrow adhesive strips. (a,c) Control cells. (b,d) Y27632-treated cells. (a,b) Cells on the control substrate. (c,d) Cells on the substrate with narrow adhesive strips. Fluorescent microscopy after staining for actin. Scale bar, 20 μm.

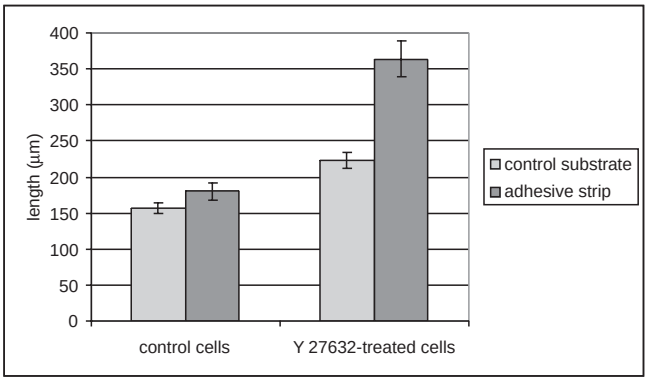


Fig. 10. Length of mouse embryo fibroblasts on control substrate and adhesive strips after Y27632 treatment (means±s.e.m.). Difference between length on strips and plane is highly significant for Y27632-treated culture but not for control culture.

and inhibitor-treated cultures, relative length on the adhesive strips was significantly greater than that on the plane.

Thus, Y27632 increased the polarization of fibroblasts and inhibited actin-bundle formation. In a similar way, the polarization of discoidal epitheliocytes induced by this drug was not accompanied by the restoration of length control. In both cell types, after Y27632 treatment, length on the adhesive strip was significantly greater than on the plane.

Discussion

Length control is not a constant characteristic of fibroblast-like cells. This feature appears or disappears reversibly in

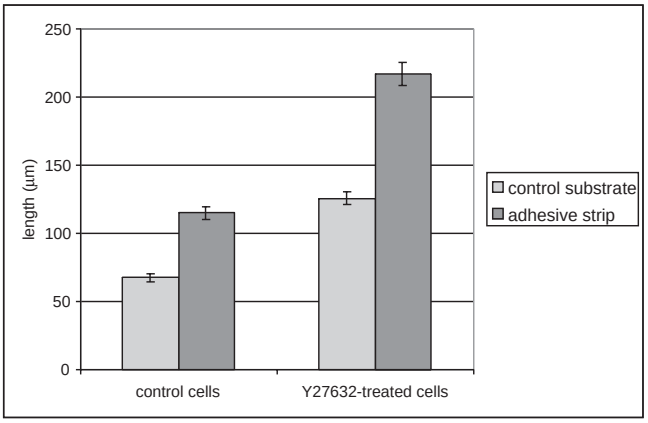


Fig. 11. Length of IAR2 epitheliocytes on control substrate and adhesive strips after Y27632 treatment (means±s.e.m.).

correlation with the appearance of polarized phenotype of cells spread on the plane. This is especially clearly shown by the experiments assessing length control at various stages of spreading. Only when the spreading cells acquire polarized shape and circular bundles disappear does their length on the plane becomes equal with that on the adhesive strip. This conclusion is also confirmed by the experiments with treatments that cause polarized cells to take discoid shapes, such as Taxol treatment of fibroblasts and, conversely, HGF/SF treatment of epitheliocytes.

Experiments show that not all polarized cells with a functioning actin-myosin system have length control; that is, their lengths are equal on the plane and on the adhesive strip. When the Rho-kinase inhibitor Y27632 inhibits contractility of this system, the cells are excessively elongated on the adhesive strip in comparison with the plane. The integrated microtubule system is also essential: length control is lost when microtubules are disintegrated by Taxol. All types of cells with circular actin bundle also have no length control. Thus, co-operation between microtubules and actin stress fibers oriented in parallel with the cell axis seem to be needed for the establishment and maintenance of length control.

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