Light-Microscopical Analysis of Focal Adhesions of Retinal Pigmented Epithelial Cells

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Retinal pigmented epithelial (RPE) cells from eyes of chick embryos form colonies in vitro in which cells at the periphery of the colony are unpigmented, undifferentiated, and well spread, while those in center of the colony are cuboidal, polygonal, and pigmented, and resemble RPE cells in vivo. The differentiated RPE cells in the center of the colony display predominantly cell-cell adhesiveness, and their microfilaments are organized in compact, circumferential rings. Undifferentiated RPE cells from the edge of the colony, in contrast, display predominantly cell-substratum adhesiveness and have numerous stress fibers spanning their cytoplasm. The well-spread RPE cells adhere to the substratum with focal contacts and unusually large focal adhesions. The focal adhesions which are typical of the spread chick RPE cells in vitro consist of several closely apposed focal contacts, arranged in a parallel fashion, which are often coalesced with each other along their sides. They occur at the termini of prominent microfilament bundles which contain F-actin and tropomyosin along their entire length. Myosin, which is also present in these bundles, however, is less abundant than actin and tropomyosin in the terminal, focal adhesion-associated parts of these bundles. On the other hand, myosin is more abundant than actin and tropomyosin outside the microfilament bundles in the bulk of the cytoplasm. Both focal adhesions and termini of microfilament bundles coincide with the restricted regions where high concentrations of vinculin, an adhesion-specific protein, are found. In contrast, an actin binding protein, spectrin, is distributed fairly uniformly throughout the entire cortex of RPE cells, and, unlike vinculin, does not seem to participate in the binding of microfilament bundles to the plasma membrane. Although extracellular matrix components laminin, fibronectin, and heparin sulfate proteoglycan are produced and deposited by the more differentiated RPE cells in the center of the colony, heparan sulfate proteoglycan has not been detected along the surface of the flat, undifferentiated RPE cells near the edge of the colony, while both laminin and fibronectin are present in very low amounts, the former along their ventral, and the latter along their dorsal cell surfaces. These data are discussed from a point of view that the formation of highly adhesive membrane-cytoskeleton complexes of the focal type in the spread, undifferentiated RPE cells is brought about by exposure of the cells to the rigid, unyielding substrata, such as glass or plastic. Expression of differentiated RPE phenotype in vitro, on the other hand, occurs when large quantities of gel-like extracellular matrix are present. The data indicate that the general organization of the adhesions in the RPE cells in vitro may be a function of both biochemical and mechanical properties of the substratum. Invest Ophthalmol Vis Sci 27:1622–1633, 1986.

The adhesion of cells to a solid substratum can be modified by changing the surface properties of cells through the action of cytoskeletal elements. Using surface reflection interference (SRI) microscopy, regions of strong interaction between cell and the substratum, known as focal contacts and focal adhesions, have been defined. Since focal contacts and adhesions are sites at the plasma membrane where microfilament bundles terminate, membrane-cytoskeleton and membrane-extracellular matrix interactions at these sites may mediate their formation and disassembly.

The notion that the focal contacts and adhesions are specialized, highly adhesive membrane-cytoskeleton complexes has been further strengthened by the discovery that several proteins are localized almost exclusively at these sites. Vinculin, a 130 kd protein, is specifically associated with the cytoplasmic side of the plasma membrane at sites where microfilament bundles terminate in focal contacts.

Because, in contrast to earlier reports, vinculin does not interact directly with microfilaments, it has been suggested that other proteins, such as talin, act as a link anchoring microfilaments to the plasma membrane in contact regions.

Several other proteins recently localized close to the cytoplasmic surface of the plasma membrane may also...
be important in maintaining the integrity of the cell cortex by linking the plasma membrane to the cytoskeleton.\textsuperscript{23,24} Because several of these proteins resemble erythocyte spectrin,\textsuperscript{2} it was thought that they may have a function in nonerythroid cells similar to that of spectrin in the erythrocyte cytoskeleton.\textsuperscript{25} Most localization studies, however, have not produced clear images documenting the relationship between the distribution of the non-erythroid spectrins and the major cytoskeletal elements or the adhesions patterns of these cells.

Proteins of extracellular matrix (ECM) also play an important role in promoting and regulating cellular adhesiveness.\textsuperscript{26-29} Their distribution in relation to the cell-substratum adhesion patterns, however, is not clear at present. Distribution of fibronectin in relation to focal contacts and adhesions has been the subject of considerable controversy.\textsuperscript{28} In fact, if association of various ECM proteins with these adhesive structures is highly dynamic, it may be difficult to determine whether they are present in or absent from these structures.

Although focal contacts have been extensively studied in fibroblasts, these and related forms of adhesion have not been examined in epithelial cells in detail. Such information, however, is required to allow generalization about their structure, function, and interactions with the cytoskeleton and ECM. Also in fibroblasts, the vulnerability of focal contacts to fixation artifacts due to their small size\textsuperscript{30} has complicated attempts to correlate the distribution of cytoskeletal or ECM proteins with their adhesion patterns. In contrast to fibroblasts, well-spread retinal pigmented epithelial (RPE) cells of the chick embryo display unusually large focal adhesions when viewed by SRI microscopy.\textsuperscript{8,9} In which comparison between the distribution of various ECM and cytoskeletal components and the cell-substratum adhesion patterns can be made more easily. Although the adhesion of RPE cells to the substratum in vitro is probably only remotely related to the RPE-Bruch's membrane adhesion in vivo, the basic mechanisms underlying both types of adhesion are likely to be similar. Thus, we are of the opinion that insight into the mechanism of RPE adhesion in vitro, besides providing new information on the role of cytoskeletal components in mediating cell adhesion, should help in understanding the in vivo events occurring in normal, pathological, and experimentally changed conditions.

In the present study, we have sought to determine the nature of the major protein components on the intra- and extracellular side of focal adhesions in RPE cells by immunofluorescence microscopy and SRI microscopy. Using a specific probe for F-actin and antibodies against myosin, tropomyosin, vinculin, and spectrin, and fluorescence microscopy, we have studied the distribution of these cytoskeletal components in the RPE cells. We have also studied the intra- and extracellular distribution of components of ECM produced by the RPE cells in vitro using antibodies against laminin, heparan sulfate proteoglycan, and fibronectin, and fluorescence microscopy. The localization of these cytoskeletal and ECM components have been correlated with the adhesion patterns in the same cells by visualized SRI microscopy. By this integrated approach, we have tried to gain more insight into the organization of cell-substratum adhesions in the RPE cells in vitro, as well as to compare them with the adhesive structures observed in other cell types. We find 1) that the distribution of vinculin coincides precisely with the distribution of focal adhesions, 2) that any of the ECM components examined are concentrated in, or excluded from, the focal adhesions, and 3) that, of the three proteins present in the bundles of microfilaments, actin and tropomyosin extend to the ends of these bundles, whereas the amount of myosin is greatly reduced in the regions of the bundles where focal contacts and adhesions are found. On the basis of the present observations and available data, we discuss biomechanical aspects of the spatial reorganization of microfilament-based cytoskeleton occurring during growth and differentiation of RPE cells in vitro.

Materials and Methods

Sheets of retinal pigmented epithelium were explanted from the eyes of 8-day-old chick embryos and established in tissue culture as described.\textsuperscript{9} Explants on glass coverslips were maintained in a humidified incubator in a 5% CO\textsubscript{2} atmosphere at 37°C in alpha minimal essential medium supplemented with 7% heat inactivated fetal calf serum (Gibco, Burlington, Ontario) and containing 100 IU penicillin, 0.25 µg fungizone and 100 µg streptomycin (Gibco, Burlington, Ontario) per ml.

The characterization of antisera used in the present work, demonstration of their specificity for appropriate antigens, that is, myosin and tropomyosin,\textsuperscript{5} vinculin and spectrin,\textsuperscript{31,32} and for ECM components, laminin, fibronectin, and heparan sulfate proteoglycan,\textsuperscript{25} in cultured chick RPE cells and immunofluorescence microscopy procedures with their appropriate controls have been published previously. The use of fluorescent probes, NBD-phallacidin (for visualization of F-actin), and FITC-WGA (for visualization of lectin binding materials) has been described for the RPE cells.\textsuperscript{9,24,30}

For immunofluorescence microscopy cells on coverslips were fixed in 3.7% formaldehyde in PBS for 5–10 min, extracted with RST X-100 solution (0.1% Triton X-100 in buffer containing 0.1 M PIPES, 0.001 M EGTA, 4% (w/v) polyethylene glycol 6000, pH 6.9) for 3 min, washed in PBS for 10 min, and then incubated
for 30 min at room temperature with the appropriate antiserum. Afterward, the cells were washed in PBS for 10 min and incubated for another 30 min with either TRITC- or FITC-conjugated secondary antisera (Miles, Rexdale, Ontario) diluted 1:50 in PBS. Finally, the coverslips were washed for 10 min in PBS and mounted on slides in PBS containing 0.25% 1,4-diazabicyclo(2,2,2)-octane (Polysciences, Markham, Ontario) and 0.002% para-phenylenediamine to prevent photobleaching. 

In some experiments, microfilaments were visualized with SRI microscopy after Coomassie Blue staining (SRI-CooB technique). To analyze the distribution of F-actin with respect to adhesion patterns, the cells were fixed in glutaraldehyde (2% for 60 sec), followed by formaldehyde (3.7% for 10 min), and RST X-100 solution (30 sec), and then incubated with 0.3 μM NBD-phallacidin (Molecular Probes, Junction City, OR) for 30 min at room temperature. This method of fixation has been found to give excellent preservation of adhesion patterns, while permitting the concomitant use of the actin-specific fluorescent probe NBD-phallacidin. 

Fluorescence and SRI microscopy was performed using a Photomicroscope II (Zeiss, Oberkochen, FRG) as described. Photographs were taken using Ilford XP1-400 and Kodak Tech Pan 2415 films for fluorescence and SRI microscopy, respectively.

Results

Embryonic chick RPE cells form colonies in vitro in which the cells occupying the center of the colony are polygonal, pigmented, and resemble closely RPE cells in vivo, while those towards the colony edge are progressively flatter, less pigmented, and display features characteristic of undifferentiated epithelial cells. When cells at the perimeter of the colony (Fig. 1a) are examined by SRI microscopy, focal, close, and far contacts are evident, as well as unusually large focal adhesions (Fig. 1b). The association of focal contacts and focal adhesions with actin-containing microfila-

ments can be demonstrated directly by staining the latter with the F-actin-specific fluorescent probe, NBD-phallacidin. Fluorescence microscopy of RPE cells reveals that the focal contacts and adhesions (Fig. 2a, c) are always associated with the ends of microfilament bundles (Fig. 2b, d). A closer examination of the focal adhesions of RPE cells (Fig. 2c) shows that they are aggregates of individual black streaks that are apposed to each other along their sides. Since, in the same part of a cell, each of these streaks in the focal adhesion visualized with SRI microscopy is associated with an end of a microfilament bundle visualized with fluorescence microscopy after NBD-phallacidin staining (compare Figs. 2c with 2d), it is clear that each of them correspond to a single focal contact.

Immunofluorescence microscopy of RPE cells shows that their microfilament bundles contain both tropomyosin (Fig. 3b) and myosin (Fig. 3d). A comparison of SRI and immunofluorescence images of the same cells indicates, however, that although tropomyosin is abundant in terminal regions where microfilament bundles are associated with focal adhesions (Fig. 3a, b), myosin seems to be partly excluded from these regions (Fig. 3c, d). To compare the distribution of actin, myosin, and tropomyosin in relation to the adhesion patterns in the same RPE cells, we have used double immunofluorescence in combination with SRI microscopy. A comparison of distribution of tropomyosin (Fig. 4b) with the distribution of myosin (Fig. 4c) within the focal adhesions (Fig. 4a) of the same cell, and a comparison of distribution of actin (Fig. 4e) with the distribution of myosin (Fig. 4f) within the focal adhesions (Fig. 4d) of another RPE cell, confirms that, when compared to actin and tropomyosin, myosin is less abundant in those parts of microfilament bundles which are associated with the focal adhesions of RPE cells. Myosin, however, appears to be more abundant in the bulk of the cytoplasm within the cell body.

Staining of RPE cells with antisera against the two proteins associated with the cell cortex, vinculin and spectrin, reveals that, while the former is highly concentrated in focal contacts and adhesions (Fig. 5), the
Fig. 2. SRI images of adhesion patterns (a, c) and NBD-phallacidin fluorescence showing F-actin (microfilaments) distribution (b, d) in RPE cells. Most of the prominent microfilament bundles terminate in focal adhesions (compare a with b). At higher magnification, it becomes clear that each of the focal contacts (small, black streaks in c) is associated with a fine bundle of microfilaments (small white streaks in d), whereas focal adhesions (large, black areas in c) are associated with the termini of prominent ones (large white areas in d). Scale div. = 5 \mu m (a, b), 2 \mu m (c, d).

Fig. 3. The patterns of adhesion (a, c) and the distribution of tropomyosin (b) and myosin (d) in RPE cells. Note that, while tropomyosin staining is intense along the entire length of microfilament bundles, including their focal adhesion-associated termini, myosin staining becomes weaker in the regions associated with focal adhesions. Scale div. = 5 \mu m.
Fig. 4. The patterns of adhesion (a, d) and double-label visualization of microfilaments using antisera to tropomyosin (b) and myosin (c, f) or NBD-phallacidin (e). Comparison of a, b, and c demonstrates that, while the anti-tropomyosin staining is intense and concentrated along the microfilament bundles (b) extending to the ends of focal adhesions (compare with a), the anti-myosin staining of the same cell (c) is much more diffuse and concentrated on parts of microfilament bundles in the cell body, and markedly diminishing towards the peripheral regions (compare with b) where the adhesion foci are located. d, e, and f show a similar difference in distribution between actin (e) and myosin (f). When compared to the adhesion pattern (d), the actin staining extends to the ends of microfilaments bundles, where they end in adhesion foci, while myosin seems to be concentrated in the cell body and is much less abundant towards the cell periphery. Arrows point to the same areas in a, b, and c, and in d, e, and f. Scale div. = 5 μm.

Fig. 5. SRI (a) and immunofluorescence (b) images after staining of the same RPE cell from the edge of the colony with vinculin antiserum. Note that all the focal adhesions (a) correspond to regions of bright fluorescence (b), indicating heavy accumulation of vinculin in those areas. Scale div. = 5 μm.
Fig. 6. SRI (a) and immunofluorescence (b) images after staining of the same RPE cell with spectrin antiserum. Comparison shows that the distribution of spectrin (b) is not related to the distribution of focal contacts and focal adhesions visualized by SRI microscopy (a). Scale div. = 5 μm.

The latter is distributed much more evenly over the entire cell cortex and bears no obvious relationship to these sites (Fig. 6). In experiments of triple-labelling of the same RPE cells, spectrin was visualized by immunofluorescence with spectrin antiserum (Fig. 7b), lectin-binding materials were visualized by fluorescence microscopy with fluoresceinated wheat germ agglutinin (Fig. 7c), and microfilaments along the adhesive ventral surface were visualized by SRI-CooB microscopy (Fig. 7d). The distribution of focal contacts and adhesions in the same cells was visualized by SRI microscopy (Fig. 7a). The results of these experiments demonstrate that neither spectrin nor lectin-binding components of the cortical region co-distribute with focal contacts and adhesions, or with the contact-associated termini of microfilament bundles.

Immunofluorescence microscopy with antisera against two components of ECM, laminin and HSPG, reveals that, in fixed and permeabilized cells, both laminin (Fig. 8b) and HSPG (Fig. 9b) are present intracellularly. Their distribution, however, is not related to patterns of adhesion visualized in the same cells by SRI microscopy (Figs. 8a and 9a, respectively). Staining of cells which were not permeabilized reveals very small amounts of extracellular laminin (Fig. 8d) and no extracellular HSPG (Fig. 9d) in well-spread RPE cells.

Fig. 7. The same group of RPE cells observed by SRI microscopy (a) to show the adhesion pattern, by fluorescence microscopy (b) to visualize the distribution of spectrin after staining with antiserum to spectrin and TRITC-conjugated secondary antibodies, by fluorescence microscopy (c) after staining with FITC-conjugated wheat germ agglutinin to show the distribution of lectin-binding materials, and by SRI-CooB microscopy (d) to visualize the adhesion-associated termini of microfilament bundles. Note that, while there is a high degree of correspondence between the adhesion patterns (a) and termini of microfilament bundles (d), there is little or no correspondence between these structures and the distribution of spectrin (b) and lectin-binding materials (c). Scale div. = 5 μm.
Staining of fixed and permeabilized cells with antibodies against fibronectin demonstrates the presence of very small amounts of this protein in the form of fine aggregates and fibers located at the upper cell surface of the well-spread RPE cells (Fig. 10).

**Discussion**

**Focal Adhesions: A Definition**

Retinal pigmented epithelial cells obtained from the eyes of chick embryos form colonies in vitro, in which...
Fig. 10. The patterns of adhesion (a) and the distribution of fibronectin (b) in the well-spread RPE cells. Fibronectin in these permeabilized cells is seen mostly along the upper cell surface (b) in the form of small aggregates and fine fibrils. Few regions with very weak, diffuse cytoplasmic staining can also be seen. This staining pattern does not correspond to the adhesion patterns seen in the same cells in a. Scale div. = 5 μm.

cells at the periphery of these colonies express an undifferentiated, well-spread morphology and develop extremely large areas of cell-substratum adhesion.\(^9\),\(^32\) These adhesions have been classified as focal on the basis of: a) their black image in SRI microscopy, the contrast of which is not affected by changes in either the wavelength of the incident light or the refractive index of the immersion medium;\(^8\) b) their association with the termini of microfilament bundles; and c) their ability to be labelled with antiserum against vinculin, a protein specific for adhesions of the focal type.\(^1\),\(^2\),\(^3\),\(^12\)-\(^15\) Each of the focal adhesions of RPE cells consists of a number of laterally associated focal contacts appearing as black streaks by SRI microscopy.\(^8\) Occasionally, a number of these black streaks can be resolved within a focal adhesion and assigned to the individual fine microfilament bundles visualized with fluorescence microscopy after NBD-phallacidin staining. Therefore, we conclude that the focal adhesions of RPE cells comprise a number of laterally apposed focal contacts, which are often coalesced with each other along their sides. The additional characteristics of focal adhesions is that they are always more permanent than focal contacts.\(^6\)-\(^8\)

Cytoskeleton and Focal Adhesions

Although the focal contacts and adhesions are invariably associated with bundles of microfilaments,\(^1\) the identity and properties of proteins anchoring actin filaments to the plasma membrane in these areas are poorly understood. Among the potential linking proteins, vinculin, a 130 kd protein found in focal-type adhesions,\(^1\),\(^2\),\(^12\)-\(^13\) is well known, and its localization\(^14\),\(^15\) and interaction with both actin\(^16\)-\(^19\) and the plasma membrane\(^20\) have been thoroughly investigated. It has been suggested that spectrin may also link the plasma membrane to the microfilaments.\(^23\) The finding that the distribution of spectrin does not follow the changes in the distribution of microfilaments during the acquisition of the in vivo morphology by RPE cells in vitro,\(^32\) and that does not codistribute either with microfilament bundles or with focal adhesions in the well-spread, undifferentiated cells remaining at the margins of RPE colonies suggest that, at least in RPE cells, it does not bind the large bundles of microfilaments to the plasma membrane. Microfilaments, however, are also present in the form of a fine cortical meshwork;\(^2\) thus, from its distribution throughout the cell cortex, spectrin could very well be involved in binding the microfilaments of this meshwork to the cell membrane.

Recently, we have shown that, in contrast to fibroblastic cell lines, in the RPE cells, spectrin does not codistribute with lectin-binding components of the outermost part of the cortical cytoskeleton.\(^24\) Thus, it is highly probable that spectrin may have different roles in different cell types. Furthermore, immunoprecipitation of spectrin within living fibroblasts or epithelial cells by microinjection of anti-spectrin antibodies does not affect their shape, motility, or the distribution of microfilaments.\(^38\),\(^39\) These findings suggest that spectrin in the cortex of nonerythroid cells is not required for maintaining adhesiveness, cell shape, and the integrity of the actin-containing cytoskeleton.

Although contact with the substratum is a necessary prerequisite for cellular translocation, it is close contacts which are important for fast migration, while focal contacts and adhesions are crucial for the spreading of cells across the substratum.\(^6\),\(^7\),\(^40\)-\(^41\) Because the depletion of myosin has been observed in actin-rich regions near cell-cell contacts in fibroblasts,\(^42\) as well as the RPE cells,\(^43\) it seems possible that microfilaments, together with their associated proteins, play more of a structural rather than a contractile role in the contact regions. Because myosin is also less abundant in isolated focal contacts which have been subsequently analysed by SDS-polyacrylamide gel electrophoresis,\(^44\) it is unlikely that some other component(s) block the access of the antibody to the ends of microfilament bundles in the focal adhesions of RPE cells to account for the reduced staining. These earlier observations, together with the relative depletion of myosin in parts of microfilament
bundles associated with the focal adhesions, observed by us in the RPE cells, suggest that both cell-cell and cell-substratum contacts of the focal type are constructed according to a similar architectural plan. This notion is further strengthened by the finding that both types of focal contacts employ a similar, vinculin-dependent mechanism for anchoring the microfilament bundles to the plasma membrane. Caution has to be exercised in constructing the above analogy, however, because it has been shown that a new 135 kd protein, which is specifically associated with cell-cell focal contacts, is not present in the cell-substratum ones.

ECM and Focal Adhesions

The chick RPE cells in vitro produce and deposit ECM, in which collagen type IV, laminin, HSPG, and fibronectin have been detected. The importance of ECM is the regulation of cell structure and function, and the role of proteins such as laminin and fibronectin in this process, have recently become increasingly apparent. Laminin receptor has been recently identified and appears to function as an actin-binding protein. It was conceivable, therefore, that this protein would be located in the region of focal adhesions. In the present paper, we demonstrate that the well-spread, undifferentiated cells from the edges of the RPE colonies have extensive focal adhesions at sites which lack detectable amounts of laminin, as well as fibronectin, or HSPG. Of the three, laminin and HSPG are produced by the spread RPE cells, as shown by the heavy cytoplasmic labelling of these cells with the corresponding antibodies. It seems, however, that the deposition of fibrillar laminin and HSPG, together with the production and deposition of fibronectin, is carried out by the central, more differentiated RPE cells which lack focal adhesions. Similar results have been recently obtained by Crawford and Vielkind. Thus, it seems that, while the packed, polygonal cells from the colony center are adhering to a thick gel-like pad of ECM, the spread, peripheral RPE cells are exposed to a nearly bare glass, enabling them to form focal contacts and adhesions. We have demonstrated previously, however, that even the tightly packed, cuboidal, and differentiated RPE cells are capable of developing cell-substratum adhesions of the focal type, but only before extensive ECM is deposited. Therefore, all the RPE cells, irrespective of their shape and degree of packing in a sheet, can establish focal-type adhesions, provided they are exposed to a rigid, unyielding substratum, such as glass or plastic. A gel-like, yielding pad of ECM provides a highly deformable substratum which does not promote strong cell-substratum adhesions. Thus, it seems justified to assume that, in RPE cells in vitro, a substratum deformability and the composition and quantity of ECM which has been deposited should be considered as major factors determining the interactions of the cells with substratum and, subsequently, the spatial organization of the cytoskeleton.

Biomechanical Aspects of Cytoskeletal Organization in RPE Cells

Spread cells can sense an anisotropy in substratum elasticity and react to it, as well as reacting to an elastic deformation externally applied to the substratum. The behaviour of cells as “elastometers” is facilitated by the fact that the spread cells themselves are under tension which has slow (contractile) and fast (elastic) components. Necessarily, in a spread cell, the tension is being transmitted to the substratum and, when the substratum is elastic enough, it becomes deformed as to reflect the forces exerted on it by the adherent cell. This is based on the assumption that, at any moment, cell tractional forces are in mechanical equilibrium with the elastic forces operating within the substratum. Mechanically, a spreading cell, while increasing its circumference, generates centripetal tension transmitted to the substratum by adhesive “patches” periodically distributed along the cell circumference. According to Fleischer and Wohlfarth-Bottermann, the development of steady tension, that is, an *isometric contraction*, promotes the formation of microfilament bundles, which, in turn, are contractile. The formation of contractile microfilament bundles breaks down the circumferential arrangement of adhesions, and a spread cell becomes irregularly stretched between the few strongest points of attachment to the substratum. In this simplified, mechanistic model, the arrangement of stress fibers in a fully spread cell has been proposed to at least partly reflect the lines of a tension field generated by cellular contractile activity and spatially restricted by adhesions to the substratum.

From a biomechanical point of view, a single epithelium is a two-dimensional association of tightly adherent cells which actively contract, thus forming a tensile sheet which rests on a viscoelastic support, i.e., basement membrane. Both the RPE in vivo and the differentiated RPE in vitro follow this scheme, being a tightly packed cell layer resting on a complex three-dimensional basement membrane which contains collagen type IV, laminin, HSPG, and fibronectin. The structural basis of the generation of tension in the RPE cell sheet seems to consist of the cytochalasin B-sensitive circumferential rings of microfilaments, which have been shown to be contractile both in situ and after isolation, and which, in addition to actin, contain a set of regulatory proteins, such as myosin and tropomyosin. The cortical ring of microfila-
ments circumscribes the differentiated RPE cells at the level of a belt of zonulae adherents, and seems to be attached to the cell membrane by electron-dense amorphous material.\textsuperscript{36,68,69}

In the present paper, we have shown that, in the stress fibers in the spread RPE cells near the edge of the colony, proteins are spatially segregated along the length of a fiber in such a manner that the "attachment" proteins concentrate in their distal (membrane-facing) parts, while the "contractile" proteins predominate in the proximal (cytoplasmic) parts of the fiber. The situation, thus, is analogous to the one in the cortical rings in the differentiated RPE cells in the center of the colony (or in vivo\textsuperscript{70}), where the same proteins are spatially segregated across the width of a ring, again, with the "attachment" proteins concentrated on its outer (membrane-facing) side, while the "contractile" proteins predominate on inner (cytoplasmic) side of a ring.\textsuperscript{51} It is, therefore, conceivable that the linear stress fibers and the circumferential rings of microfilaments are homologous structures, both contractile and contracting isometrically, and attached to the cell membrane by means of "attachment" proteins present at their membrane-facing sides. It should be stressed that there are structural differences between these two forms of microfilament arrangement; for example, while stress fibers attach to the cell membrane in focal contacts and adhesions through vinculin-talin complex, circumferential rings of microfilaments present in areas of cell-cell contact presumably attach to the plasma membrane through vinculin-135 kd protein complex.\textsuperscript{71}

Both stress fibers and circumferential rings of microfilaments, however, may have their origin in contractile structures, which have been generating tension and/or have been subjected to the tension.

The proposed homology between linear stress fibers and circumferential microfilament rings is far from being obvious, from their respective spatial distributions. However, the striking difference between these two spatial arrangements of microfilaments (linear vs circumferential) can be explained, according to the mechanistic model presented above, by the different spatial distribution of adhesion sites (planar array of focal contacts and adhesions vs belt of zonulae adherents) associated with the respective microfilament arrangements. By restricting the shape of individual cells, preventing thereby the isometric contraction from entering into the isotonic phase, the latter promoting microfilament bundle disassembly,\textsuperscript{60} the adhesion sites, and their spatial arrangement can be considered a major factor in shaping a geometry of the microfilament-based cytoskeleton. To account for the above observations, a mechanistic hypothesis has been proposed,\textsuperscript{72} in which the organization of RPE cells during growth and differentiation in vitro is thought to be a function of cell adhesiveness and the substratum deformability, where both the linear and circumferential arrangements of microfilaments are proposed to reflect the lines of a tension field generated by the cell's contractile activity and spatially restricted by the sites of adhesion.

**Key words:** cell-substratum adhesion, cytoskeleton, extracellular matrix, differentiation, RPE, tissue culture

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