Quantitation of actin polymerization in two human fibroblast sub-types responding to mechanical stretching

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Summary

To study early reorganization of the cytoskeleton in response to physical forces, human gingival and periodontal ligament fibroblasts were cultured on flexible plastic substrate and stretched by mechanical deformation of the substratum. F-actin was measured by quantitative spectrofluorimetry of FITC-phalloidin-stained cells. Fluorescence due to FITC-phalloidin was reduced stoichiometrically by co-incubation with unlabeled phalloidin. Increases in stretch-induced fluorescence were blocked by pre-incubation of cells with cytochalasin D. Compared to baseline values, F-actin in gingival fibroblasts was reduced by 50% at 10 s after stretching but was increased more than 100% at 50 s after stretching. Increases of F-actin were also dependent on the amount of deformation of membrane: 3.3% stretching produced the largest increase and 9.2% stretching produced the least. Stretch-activated actin polymerization was blocked by pre-incubation of cells with 500 ng/ml pertussis toxin or 10 mM EGTA. Compared to gingival cells, periodontal ligament fibroblasts exhibited twice the total actin per cell and three times the baseline F-actin per mm², however, there was no increase in fluorescence after stretching. In both cell types, stretching caused increased ruffling activity and no detectable shape changes at 60 s, but at 180 s the cells shortened and pseudopods retracted. The microfilament system of fibroblasts exhibits rapid dynamic responses to mechanical deformation prior to cell shape changes and appears to be dependent on the cell type.

Key words: actin, fibroblasts, stretching.

Introduction

Rearrangements of an actin-rich, sub-membrane cortex (Bray and White, 1988; Janmey et al. 1990) provide the basis for many dynamic cellular functions including motility (Spooner et al. 1971; Korn, 1978), chemotaxis (Howard and Meyer, 1984), phagocytosis (Grinnell and Geiger, 1986) and cell adhesion (Wang, 1984; Southwick et al. 1989). Cortical actin also contributes to the elasticity of cells responding to environmental changes (Petersen et al. 1992) and permits cells such as fibroblasts to generate forces greater than are needed for maintenance of cell shape or locomotion (Harris et al. 1981; Bell et al. 1979; Bellows et al. 1981). However, the mechanisms by which specific shape changes in non-muscle cells are mediated and by which actin rearrangement is regulated are not completely understood. It is generally believed that microfilament–membrane interactions (Geiger, 1983), regulation of actin polymerization by actin-binding proteins (Korn, 1982; Pollard and Cooper, 1986), and ATP and divalent cation binding to actin (Carlier, 1991) are crucial control elements in shape and force-generating phenomena (Stossel, 1988; Carraway, 1990).

Coordinated regulation of shape changes with rearrangement of the cytoskeleton has been observed in the microfilament arrays of osteoblasts (Buckley et al. 1988), endothelial (Sumpio et al. 1988) and epithelial cells (Brunette, 1984; Kolega, 1986), responding to mechanical deformation and in the rapid pseudopodial extension of amoebae (Condeelis et al. 1988) and neutrophils (Wymann et al. 1990) responding to chemotactic agonists. These model systems have provided insights into the role of cortical actin in transmembrane signalling (Stossel, 1988) and how stimuli are translated into actin assembly and shape changes. Although a great deal of data on the regulation of actin polymerization have been gathered using the chemotactic response of suspended neutrophils to agonists (e.g. see Howard and Meyer, 1984; Howard and Oresajo, 1985; Norgauer et al. 1989; Wymann et al. 1990), there is much less known about early dynamic events in microfilament systems of substratum-attached cells responding to mechanical deformation.

To study actin polymerization in the early response of fibroblasts to mechanical deformation, cells were attached to flexible substrata and F-actin was quantitated fluorimetrically as modified from the original method developed for neutrophils (Howard and Meyer, 1984; Southwick et al. 1989). On the basis of a technique adapted from Brunette (1984), precise and reproducible deformations of cells were made, enabling quantitative assessments to be made of the relationships between cell membrane deformation and actin polymerization. To assess the possible role of fibroblast heterogeneity (Narayanan and Page, 1983) in the response of cells to stretching, two different fibroblast sub-types with distinct and separate phenotypes were compared, and the dependence of actin assembly on GTP-
binding regulatory proteins (Shefeyk et al. 1985) and extracellular calcium (Downey et al. 1990) was investigated.

Materials and methods

Cell cultures

Fibroblasts derived from human gingiva and human periodontal ligament were obtained using the methods of Brunette et al. (1976) and were grown to subconfluence in T-75 flasks (Falcon, Becton Dickinson, Mississauga, ON) containing minimal essential medium (a-MEM) supplemented with antibiotics (0.17% penicillin V and 0.1% gentamycin sulfate) and 15% foetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. For all experiments, cells between the third and tenth passage were used. Approximately 14 h before each experiment, cells were trypsinized and plated at 10^5 cells in 60 mm dishes with a flexible, hydrophilic plastic growth surface (Petriperm, Bachofer GmbH, Reutlinger, FRG). This low plating density successfully reduced the likelihood of cell-to-cell contacts, which would add additional complexity to the study of cytoskeletal dynamics after membrane deformation (Kolega, 1986).

Application of mechanical stretching

The Petriperm dishes were mechanically stretched by placing them over convex plastic shapes at room temperature. Control dishes were placed on flat plastic shapes fitted to the base of the dish. A clamp was fabricated that permitted rapid (<1 s) deformation of the base of the Petriperm dish to conform to the surface of six different interchangeable convex shapes. On the basis of direct linear measurements of fixed distances inscribed into the center of the membrane, the curvature of these plastic shapes stretched the membrane of the Petriperm dishes 0, 1%, 1.2%, 3.3%, 6.8% and 9.2%, respectively. These measurements were consistent with geometric predictions of linear distance on the basis of the height and width of the convex shapes. Dishes were removed from the incubator and stretching was carried out immediately under a laminar flow hood using sterile conditions.

Determination of cellular F-actin and morphometry

The application of stretching was timed and the cells were fixed at the end of the stretch period by the addition of 3.7% formaldehyde. The cells were fixed for 15 min at room temperature and stained with fluorescein-phalloidin (FITC-phalloidin; Sigma, St Louis, MS; Wymann et al. 1990) at a concentration of 4 × 10^{-6} M in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Nonidet. Pilot experiments conducted initially with NBD-phallacidin demonstrated excessive photobleaching of the fluorochrome that complicated fluorescence measurements by concentrating and superimposing fluorescent intensities in a very small measurement area and thereby induce non-linearity into fluorescence measurements. Control cultures were treated with vehicle alone. To examine the possibility of stretch-induced cell death, some dishes were incubated with propidium iodide (10 mg ml^{-1} PBS) immediately after stretching and without fixation. To determine the importance of extracellular calcium on actin assembly after stretching, cells were incubated with normal medium containing no exogenous Ca^{2+} and 10 mM EGTA 5 min before stretching. To study the role of GTP-binding proteins in stretch-activated actin assembly, 500 ng ml^{-1} pertussis toxin (List Biologicals, Campbell, CA) was added to cultures 2 h before stretching.

Total actin determination

Estimates of total actin were obtained by SDS-PAGE (Bray and Thomas, 1975) and densitometric quantitation of 41 × 10^{3}M, bands co-migrating with actin standards (Spudich and Watt, 1971). Data from 4 dishes each of gingival and periodontal ligament fibroblasts at 10^{5} cells per dish were analyzed.

Statistical evaluation

Fluorescence intensity was measured in photometer units and expressed as the median (±inter-quartile range). Comparisons between different groups were examined with non-parametric Wilcoxon tests. Cell length, cell area and actin content were expressed as mean ± standard deviation and were tested with Students t-test. Alpha was set at 0.05. In some experiments, fluorescence intensity was sampled as a function of cell nuclei number and this relation was examined by Pearson’s correlation. Fluorometric quantitation of F-actin in stretched cells was assessed in 12 different experiments using both gingival and periodontal ligament fibroblasts.

Results

Fluorescence fading over time demonstrated a 1–3% reduction between 0 and 30 s and a 20–30% reduction between 30 and 90 s. These data indicated that fluorescence intensity was stable during the first 30 s during which data were acquired. Changing the focus to 2 μm below or below the focal plane of the cells contributed 5% to the variation in measurements (± 2 photometer units). Autofluorescence comprised 28% of the total fluorescence signal and was subtracted from the total fluorescence signal for each experimental condition. The fluorescence...
due to FITC-phalloidin was reduced 10-fold when cells were co-incubated with 4×10⁻⁶ M unlabelled phalloidin (i.e. ten times the amount of unlabelled phalloidin) thus indicating specific binding. Other experiments conducted with propidium iodide staining of nuclear DNA in fixed cells demonstrated that the fluorescence measured by the fluorimeter was linearly related to the number of propidium iodide-stained cell nuclei (r=0.98). Therefore the fluorimetry measurements were directly proportional to the concentration of fluorescent species and in measurements of FITC-phalloidin at the concentrations used here, are proportional to the concentrations of cellular F-actin (Howard and Meyer, 1984; Southwick et al. 1989).

Both gingival and periodontal ligament fibroblasts were tested for viability by staining with propidium iodide after stretching for 60 s with the 9.2 % stretcher. At no time were non-viable cells found to be greater than 4 % of the population, indicating that fluorescence measurements were made on viable cells with intact cell membranes after stretching.

Gingival fibroblasts exhibited large increases in F-actin as a function of the amount of stretch after 60 s: 3.3 % stretch produced the greatest fluorescence intensity per μm² (P<0.01) and 9.2 % the least (P>0.2; Fig. 1). The increase in fluorescence was not obviously restricted to any one part of the cell (e.g. lamellipodium or stress fibers), indicating that the increased F-actin was due to a global cellular change in cortical actin. Photometry measurements of isolated portions of cells including lamellipodia and cell bodies also revealed no selective site of increased fluorescence, since very similar increases in fluorescence were observed after 3.3 % stretch in both whole cell measurements and in isolated regions (P>0.2).

Variations in the amount of stretch were also associated with alterations in cell area and length. After 60 s of stretch, the cell area increased slightly as the percentage stretch was increased, but cell length tended to be reduced. Image analysis data of Toluidine Blue-stained cells demonstrated identical changes in cell length and area, indicating that morphometric data obtained from FITC-phalloidin-stained cells were accurate. Owing to the wide variations between cells of length and area measurements, individual computations of area:length ratios were computed. These data demonstrated a linear increase of area:length as a function of % stretch (r=0.85), indicating that cells were elongated in proportion to the amount of stretch. Examination of individual cells by videocine microscopy (3 frames s⁻¹) over the first 60 s of stretch confirmed that at 1 % stretch no detectable change in cell area or length occurred. However, at 180 s after stretching, large-scale changes in cell shape were evident including retraction of pseudopods and cell elongation.

Gingival fibroblasts also exhibited large variations in F-actin content that were dependent on time after stretching. After 10 s of 1 % stretch, cells exhibited up to 3-fold reductions in F-actin (P<0.01) compared to baseline (Fig. 2). However, after 60 s, there were 2-fold increases above baseline in F-actin (P<0.05) and by 75 s, F-actin had returned to values near baseline. In two other time-course experiments, identical patterns of F-actin dynamics were seen in gingival fibroblasts.

On the basis of these observations, additional experiments were conducted in which cells were stretched 1 % for 60 s and then allowed to relax. This amount of stretch did not exceed the elastic limit of the flexible membrane, ensuring that cells were free of deforming forces after release of tension. There was a large time-dependent

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**Fig. 1.** Gingival fibroblasts. Histograms of median fluorescence per μm² (+ interquartile range) due to FITC-phalloidin staining after 60 s of stretch; and of changes in area, length and area:length ratio (mean±standard deviation). Cells were either unstretched (0) or stretched between 1 and 9.2 % on convex plastic forms. Note the progressive increase in F-actin up to 3.3 % stretch.

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Fig. 2. Variations in F-actin content between 0 and 75 s after stretching of gingival fibroblasts. F-actin was quantitated by FITC-phalloidin staining and fluorometry.

Fig. 3. Gingival fibroblasts stretched for 60 s (1.2%) and then allowed to relax. Samples were analyzed for F-actin at 10, 20 and 30 s after relaxation.

increase in F-actin that increased over 200% above baseline values ($P<0.001$) by 30 s after relaxation (Fig. 3).

Periodontal ligament fibroblasts that were not stretched contained 298±97 more total actin than gingival fibroblasts and exhibited more than 3-fold higher baseline F-actin per $\mu m^2$ than unstimulated gingival fibroblasts ($P<0.01$; Fig. 4). However, in contrast with gingival cells, there were reductions from baseline F-actin at 60 s after stretching that were statistically significant ($P<0.05$) at 3.3% or more stretch. After stretching there was no consistent, statistically significant trend in terms of change of area, length or area:length ratio. Periodontal ligament fibroblasts consistently exhibited reductions in F-actin content at sampling times from 10–60 s after stretching.

In a separate series of experiments, eighth to tenth passage gingival fibroblasts were stretched for 60 s and, consistent with previous experiments, the F-actin increased 3-fold ($P<0.05$; Table 1). Gingival cells treated with cytochalasin D for 1 h prior to stretching exhibited no significant difference ($P>0.2$) in the amount of F-actin after a 60 s stretch. Gingival cells treated with vehicle alone exhibited no blockage of increased F-actin after stretching, indicating that cytochalasin D at the doses used here was effective in blocking the mechanism for

Fig. 4. Periodontal ligament fibroblasts. Histograms of median fluorescence per $\mu m^2$ (+ interquartile range) due to FITC-phalloidin staining after 60 s of stretch; and of changes in area, length and area:length ratio due to stretching. Fluorescence was significantly reduced at 3.3% or more of stretching.
increasing F-actin concentration in gingival cells. In contrast, and consistent with experiments described above, periodontal ligament fibroblasts exhibited a 20% reduction in F-actin after stretching. However, in unstimulated periodontal ligament cells after incubation with cytochalasin D, the F-actin was reduced 6-fold compared to untreated cells (P<0.01). After 60 s of stretching, periodontal ligament fibroblasts demonstrated no significant increase in F-actin when pre-incubated with cytochalasin D. Pre-treatment of gingival fibroblasts with pertussis toxin and EGTA blocked stretch-activated actin polymerization (Table 1). Attempts to inhibit actin assembly by blockage of ion channels with 10 μM gadolinium chloride were unsuccessful in that F-actin increased after stretching (Table 1).

Discussion

The data reported here provide quantitative evidence for dynamics of actin assembly and shape changes in short-term cellular responses to mechanical deformation. The finding that small-scale deformation of cells leads to rapid and large increases in cellular F-actin is consistent with previous morphological observations of endothelial (Sumpjo et al. 1988), osteoblastic (Buckley et al. 1988) and epithelial (Kolega, 1986) cells responding to mechanical stretching. The observation that gingival cells were elongated in proportion to the amount of stretching is generally consistent with studies of the rheological properties of isolated actin filaments. These data illustrate that, at low shear, the cytoplasm behaves mostly as a visco-elastic solid (Zaner and Stossel, 1983), while during rapid deformation or recovery from stretch, the cytoplasm may behave elastically, possibly due to the interaction of actin filament cross-linking proteins (Sato et al. 1987).

Although the kinetics of actin polymerization in gingival fibroblasts responding to stretching are remarkably consistent with the rate of polymerization in chemotactic hormone-induced amoeboid cells (Hall et al. 1988), there is a time-delay of about 3 min before large-scale, morphologically detectable changes in cell shape occur. Similar delays in cell shape changes in response to release of tension have been observed in epithelial cells (Kolega, 1986). In contrast, there is no time delay between actin polymerization and pseudopod extension of amoebae (Hall et al. 1988) or neutrophils (Wymann et al. 1990) responding to chemotactants. These data indicate that the rheological properties of the cell due to actin polymerization alone may be insufficient to account for the delayed (3 min) shape change that occurs in these cells and for variations between fibroblast sub-types (Carraway, 1990). Regulatory mechanisms including actin-binding proteins (Janmey et al. 1990) and other components of the cytoskeleton including intermediate filaments and microtubules (Inger and Folkman, 1989) are also required to produce cellular shape changes in response to membrane perturbations (Pitelka and Taggart, 1983; Geiger, 1983).

The actin polymerization response to deformation of fibroblast membranes is dependent on the amount and timing of stretch and thus exhibits stimulus-response kinetics that are dose-dependent. There also appears to be a threshold level at that further increases in stretch produce no additional increase in actin polymerization and indeed are associated with reduced F-actin. These data indicate the possible existence of membrane-bound gating systems that regulate actin monomer/polymer equilibria. Such a mechanism in which membrane deformation leads to actin depolymerization and assembly may be related to the presence of stretch-activated ion channels (Lansman et al. 1987; Stockbridge and French, 1988; Yang and Sachs, 1987, 1989; Davidson et al. 1990). Indeed, reduction of extracellular Ca²⁺ by EGTA blocked stretch-activated actin assembly, indicating that ion flow is an important mechanism in regulating actin assembly. Conceivably, actin reorganization could be regulated by large-scale shifts in intracellular calcium concentrations (Marks and Maxfield, 1990; Downey et al. 1990) that are mediated by membrane deformation of ion channels.

Consistent with the regulation of actin assembly in chemotactant-stimulated neutrophils (Shefeyk et al. 1985), stretch-activated actin assembly in fibroblasts was inhibited by pertussis toxin. These data suggest that membrane deformation stimulates actin assembly by a pathway of signal transduction operating through guanine nucleotide binding proteins (Taylor, 1990). Taken together, these findings support the existence of membrane-bound gating systems that are mechanosensitive (Sachs, 1986; Lansman et al. 1987; Davidson et al. 1990) and that are capable of regulating actin assembly.

Direct comparison of cells from 12 separate experiments using the same experimental conditions demonstrates that the fluorescence intensity of FITC-phalloidin binding per μm² in periodontal ligament fibroblasts is greater than that in gingival fibroblasts by a factor of 3. Similar data were found at all passage numbers (3–10). Total actin was also about 3-fold higher in the periodontal ligament cells. These data are consistent with those from previous in vivo (Beertsen et al. 1974) and in vitro studies (Bellows et al. 1981) demonstrating that periodontal ligament fibroblasts exhibit a phenotype rich in microfilament arrays. Therefore these cells, in spite of expected variations induced by culture (Gabbiani, 1979), exhibited wide differences in concentrations of baseline total actin and F-actin before stretching, which were readily detected by the assays used here. These data provide support for the existence of phenotypically discrete subpopulations of fibroblasts in granulation tissue (Gabbiani et al. 1978) and oral tissues (Narayan and Page, 1983) that can be discriminated on the basis of rich microfilament arrays and contractile

Table 1. F-actin in fibroblasts

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<tr>
<th>Treatment and cell type</th>
<th>Baseline</th>
<th>60 s after 1 % stretch</th>
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<tbody>
<tr>
<td>Normal medium, gingival</td>
<td>1.9 (1.2–4.3)</td>
<td>6.3 (3.0–8.5) P&lt;0.05</td>
</tr>
<tr>
<td>Cytochalasin D, gingival</td>
<td>3.4 (0–6)</td>
<td>5.0 (1.5–10) P&lt;0.02</td>
</tr>
<tr>
<td>Normal medium, periodontal ligament</td>
<td>6.2 (2–9)</td>
<td>4.6 (4.1–11.7) P=0.2</td>
</tr>
<tr>
<td>Cytochalasin D, periodontal ligament</td>
<td>1.1 (0–2.8)</td>
<td>1.5 (1.4–2.6) P=0.2</td>
</tr>
<tr>
<td>Pertussis toxin, gingival</td>
<td>3.5 (2.1–4.5)</td>
<td>3.7 (3.3–4.9) P&lt;0.5</td>
</tr>
<tr>
<td>EGTA (10 mM), gingival</td>
<td>5.6 (5.2)</td>
<td>5.6 (5.4–5.8) P&lt;0.5</td>
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</tbody>
</table>

Median and interquartile range of F-actin expressed as photometer units per μm². Cells were conditioned in indicated medium and then stretched 1 % for 60 s.
nature. Indeed, Bellows et al. (1981) have demonstrated a significantly higher ability of collagen gel contraction by periodontal ligament cells compared to gingival cells that is attributable in part to the rich microfilament arrays of the periodontal ligament cells. Actin polymerization in response to mechanical deformation also demonstrated opposite trends in these two cell types. In all experiments using periodontal ligament cells, stretching reduced F-actin. These data indicate that in fibroblasts, actin polymerization is dependent on the baseline levels of total and F-actin and also appears to be cell-type dependent. Since both types of cells were equally well spread at the low plating densities used here, and cells contacting adjacent cells were not measured, it is unlikely that gross variations in culture techniques could account for these changes.

The results of this study demonstrate that deformation of the membrane and the cytosol results in remarkably rapid changes in F-actin concentration that are dependent on cell type. The measured changes in F-actin appeared to be global and thus probably occurred in the cortical zone of the cell. Consistent with this view and by virtue of its proximity to the cell membrane, stimuli (such as mechanical deformation) acting on the cell membrane can rapidly influence the rearrangement of cortical actin (Stossel, 1989).

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