

## Cell alignment is induced by cyclic changes in cell length: studies of cells grown in cyclically stretched substrates

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Received 28 January 1999; accepted 28 April 2000

### Abstract

Many types of cells, when grown on the surface of a cyclically stretched substrate, align away from the stretch direction. Although cell alignment has been described as an avoidance response to stretch, the specific deformation signal that causes a cell population to become aligned has not been identified. Planar surface deformation is characterized by three strains: two normal strains describe the length changes of two initially perpendicular lines and one shear strain describes the change in the angle between the two lines. The present study was designed to determine which, if any, of the three strains was the signal for cell alignment. Human fibroblasts and osteoblasts were grown in deformable, rectangular, silicone culture dishes coated with ProNectin, a biosynthetic polymer containing the RGD ligand of fibronectin. 24 h after plating the cells, the dishes were cyclically stretched at 1 Hz to peak dish stretches of 0% (control), 4%, 8%, and 12%. After 24 h of stretching, the cells were fixed, stained, and their orientations measured. The cell orientation distribution was determined by calculating the percent of cells whose orientation was within each of eighteen 5° angular intervals. We found that the alignment response was primarily driven by the substrate strain which tended to lengthen the cell (axial strain). We also found that for each cell type there was an axial strain limit above which few cells were found. The axial strain limit for fibroblasts,  $4.2 \pm 0.4\%$  (mean  $\pm$  95% confidence), was lower than for osteoblasts,  $6.4 \pm 0.6\%$ . We suggest that the fibroblasts are more responsive to stretch because of their more highly developed actin cytoskeleton. © 2001 Orthopaedic Research Society. Published by Elsevier Science Ltd. All rights reserved.

### Introduction

It is well established that connective tissues adapt to changes in their mechanical environment [11,23,28,30,31]. The “trajectorial hypothesis” of Wolff suggests that trabeculae align in response to the principle compressive and tensile stresses. However, Cowin [6] appropriately noted, “The precise aspect of the strain history sensed by bone tissue is an open question”.

In cell culture, cyclic substrate deformations cause cells to change their orientations [3,4,7,8] and modify their cytoskeleton [8,25]. Cyclic stretches also modulate cell proliferation [1,2,4,22] and alter mRNA levels and synthesis of matrix proteins [5,19,20] and degradative enzymes [17]. Buck [3] reported that fibroblasts grown

on a cyclically stretched substrate aligned perpendicular to the stretch direction. Buck postulated this to be a cell avoidance response. Cyclic substrate deformations also induce alignment of osteoblasts [1], smooth muscle cells [7], embryonic myoblasts [29], and endothelial cells [8,15]. Dartsch et al. [7] found a stretch threshold between 2% and 3.5% existed for arterial smooth muscle cells, below which the cells did not exhibit an alignment response. Dartsch et al. [7,8] also noted the average angle between the cells and the stretch direction increased with increasing applied stretch. However, the aspect of surface deformation responsible for the cell alignment has not been established.

Surface deformation of planar substrates, like those that induce cell alignment, are characterized by three independent surface strains. These strains describe how a square drawn on the surface deforms. Two of the three strains quantify the length changes of the square’s sides and thus describe the conversion of the

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square into a rectangle. The third strain (shear) quantifies the departure of the corner angles from 90° and thus describes the conversion of the square into a rhombus.

An important characteristic exhibited by substrate strains, for the unidirectional stretching used in many experiments, is that the strains depend on the square's orientation with respect to the stretch direction. This direction dependence occurs even if the strains are uniform over the substrate surface. This direction dependence causes the deformations imparted to an attached cell to depend on the cell's orientation. The three substrate strains immediately beneath a cell, axial strain (length changes along the cell's major axis), transverse strain (length changes across the cells width), and shear strain (changes in angle between the axial and transverse directions) vary with cell orientation, but the strains for all orientations can be calculated if they are known for any one cell orientation [10].

In this study we investigated the response of human fibroblasts and osteoblasts to cyclic substrate deformations with the objective of determining which strain component provides the signal for cell alignment.

## Materials and methods

### Experimental design

Fibroblasts and osteoblasts were isolated from skin and cortical bone biopsies of four male patients (17–50 years) who underwent surgery for a tibial fracture repair. None of the patients had a connective tissue disease. The cells were grown in deformable culture dishes for 24 (fibroblasts) or 48 h (osteoblasts) prior to initiating cyclic stretching. The culture dishes were then cyclically stretched at 1 Hz for 24 h to 0 (control), 4%, 8% and 12% of their unstretched length using one dish for each donor, cell type, and stretch magnitude. The time-course of the alignment response was determined from parallel cultures fixed after 24, 48, and 72 h of cyclic stretch.

The distribution of cell orientations on each culture dish was described by discrete angular probability distribution [21]. This was determined by calculating the percent of cells found oriented within 5° wide intervals beginning with the stretch direction (0°) and ending at the perpendicular (90°). The Kolmogorov–Smirnov test was used to determine if two distributions were significantly different or if any distribution differed from the random cell orientation distribution [14], i.e. the uniform distribution within the range (0–90°).

### Cell isolation

Fibroblast cultures were established from skin biopsies. The biopsies were washed repeatedly in phosphate buffered saline (PBS), cut into small pieces, fixed on the surface of plastic culture dishes, and incubated in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), and 0.5% L-glutamine (termed here as DMEM-A, all compounds from Biochrom, Berlin, FRG). Osteoblast cultures were established from fibula and tibial cortical bone samples from the same donors using established methods [26]. Briefly, cells were isolated from minced bone chips, following 2 h of collagenase treatment in DMEM supplemented with 300 U/ml Collagenase (Collagenase Type IV, *Clostridium histolyticum*, Sigma Chemical Company, St. Louis, MO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C with shaking. Cultures were initiated in DMEM containing 0.2 mM Ca<sup>2+</sup> and supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin

(100 U/ml), streptomycin (100 µg/ml), and 0.5% L-glutamine (termed here as DMEM-B, all compounds from Biochrom, Berlin, FRG). Low Ca<sup>2+</sup> medium supports full expression and maintenance of the osteoblastic phenotype in primary cultures and prevents fibroblastic contamination. Both cell types were subcultured by trypsin/EDTA (0.05%/0.02%) treatment and maintained in DMEM-B. Third to fourth passage cells were used for the experiments. The cells were seeded at a density of 3000/cm<sup>2</sup> and mechanically stimulated in 5% CO<sub>2</sub> at 37°C and saturation humidity.

Culture dishes were molded from a two component silicone (RTV ME 601 A+B), Wacker Chemie, Munich, FRG). The dishes were rectangular (10 cm long × 3.5 cm wide × 1.75 cm high) with a rectangular well (6 × 3 cm) for growing cells. Before plating cells, sterilized dishes were either conditioned for seven days in DMEM containing 20% fetal calf serum or treated for 2 h with 5 µg/ml of ProNectin-F (Protein Polymer Technologies, San Diego, CA). ProNectin is an engineered polymer that contains the RGD ligand of human fibronectin. In the preliminary experiments we found no difference in cell alignment between the two conditioning methods and used the latter method for most experiments because it saved time.

### Mechanical stimulation

24 h after plating the cells, the dishes were cyclically stretched using a six station stretching apparatus (Fig. 1). One end of the culture dish was held by a stationary clamp, while the other end was clamped to a drive shaft that transmitted a linear motion obtained from a cam-follower mechanism. The magnitude of deformation was determined by the eccentricity of a 3 cm diameter circular cam. A motor (28 DT12-222P, Portescap, Pforzheim, FRG) rotated the cam shaft. Prior studies verified that the apparatus produced uniform strains on the dish culture surface [22].

To exclude fluid shear as a potentially confounding mechanism, a thin silicone membrane was attached to the dish culture surface using silicone glue at one end. The membrane was not stretched because it was attached at only one end, but the membrane surfaces were exposed to fluid shear forces while the dish was cyclically stretched. Orientation distribution of cells sampled on the membrane was not significantly different than that of control cells that were grown on the same membrane without cyclic stretching ( $P = 0.79$ ). Furthermore, the effect of fluid shear on the cell alignment can also be excluded on symmetry considerations. Specifically, fluid shear along the longitudinal centerline of the dish surface can only be along this line due to symmetry. Still, cells located along this centerline oriented away from the direction of the centerline, that is, the cells oriented away from the direction of shear flow.

### Direction dependence of substrate strains

The three substrate strains immediately beneath each cell depended on the cell's orientation relative to the stretch direction (Fig. 2). These strains were computed from the known strains of a cell oriented in the stretch direction using the coordinate transformation law for tensorial strains [10]. For a cell oriented in the stretch direction, the axial strain was equal to the applied stretch, the transverse strain was measured to be –38% of the applied stretch, and the shear strain was zero. The minus sign for the transverse strain indicates that when the substrate is lengthened along the cell axis it shortens across the cell width. The condition of zero shear for a cell oriented in the stretch direction was based upon the known loading geometry and was not directly measured. For cells oriented away from the stretch direction the axial strain decreased as the angle to the stretch direction increased and was zero at about 60°, regardless of the magnitude of the applied stretch (Fig. 2). Cells with orientations greater than 60° had negative axial strain indicating they were shortened or compressed. The peak compressive axial strain occurred for cells oriented perpendicular to the stretch direction and had a magnitude that was equal to the transverse strain experienced by a cell oriented in the stretch direction. The transverse strain for cells oriented away from the stretch direction progressively increased until it equaled the applied stretch for cells oriented perpendicular to the stretch direction. The shear strain was symmetric about the 45° direction, where it was largest, and was zero in both the stretch direction and its perpendicular.

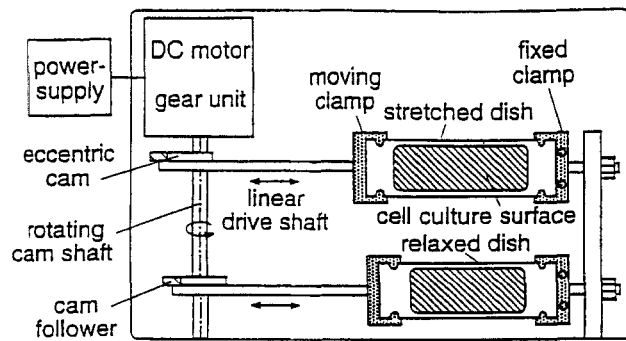


Fig. 1. A six station apparatus (two stations are shown) was used to apply cyclic stretches to rectangular silicone culture dishes. The rectangular culture dishes were held at one end by a fixed clamp and the opposite end by a moving clamp. The moving clamp was connected to a cam followed by a linear drive shaft. The extent of deformation was controlled by the eccentricity of the cams on a rotating shaft, which was driven by a DC motor through a gear reduction unit. An adjustable nut, attached to the fixed clamp, was used to remove play from the system and insure the cam follower was always in contact with the cam.

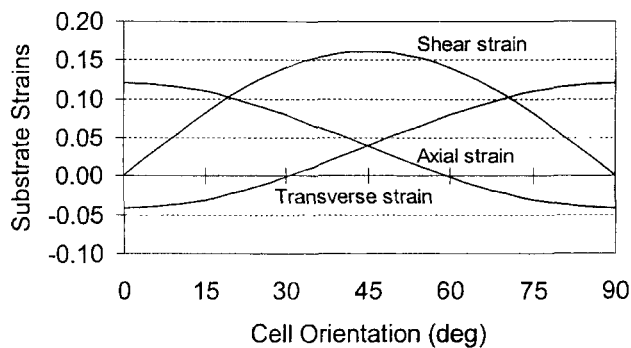


Fig. 2. The three substrate strains, axial, transverse and shear, vary with direction on the culture surface for an applied stretch (12%). Axial strain, which changes a cell's length, is largest in the stretch direction ( $0^\circ$ ) where it equals the applied stretch. Axial strain decreases as the cell's orient away from the stretch direction and is zero near  $60^\circ$ . This is the only direction where axial strain does not depend upon the applied stretch. At larger orientations, the axial strain becomes negative (compressive). The transverse strain, which changes the cell width, has the same dependence on direction as axial strain, except it is phase shifted by  $90^\circ$ . The shear strain is symmetric about  $45^\circ$ , where it is maximum, and is zero in both stretch direction and its perpendicular. Note that the curves were drawn from theory of elasticity, not from experiment.

#### Fixation and staining

Immediately after the mechanical stretching ceased, the culture supernatant was removed and the cell layers were washed twice with PBS. The PBS was removed and the cells were fixed by adding 3 ml of an ice-cold solution of methanol and formalin (90%:10%) for 1 min. The fixation solution was removed and the dishes were washed two times in double-distilled water followed by air drying. The fixed cell cultures were stained using the Giemsa method. The stretch direction was marked on the bottom of each dish using a scalpel and straight edge to scribe a thin line parallel to the side of the dish. The

bottom was then cut out and mounted on a slide for microscopic observation.

#### Data reduction and statistical analysis

The distribution of cell orientations was determined from a sample of 250–450 cells on each dish, and the total numbers of cells that were used to construct cell orientation distributions were pooled from at least two dishes from at least two donors. The cell sample was comprised of the cells located within four to six regions selected by viewing the dishes at  $50\times$  with a transmitted light microscope (Carl Zeiss, Oberkochen, Germany). The regions were selected for two reasons: (1) to avoid dish corners and edges, since they were expected to have non-uniform strain distributions; and (2) to avoid areas of high and low cell density. High cell density areas were also avoided because locally oriented groups of cells, 1–2 mm in size, occurred in the absence of mechanical stimulation. Sampling from these regions would produce bias, as the orientations of the cells in a local structure are not independent. Since the areas sampled were not selected on a random basis, we conducted additional studies, in which the sampling method used was compared with a method in which the sampled areas were randomly selected. No significant difference was found between the two sampling methods ( $P = 0.9$ , Kolmogorov-Smirnov test). We also re-sampled data from one experiment using the random sampling approach and again found no difference between the two sampling methods.

Within each sampled area, cell orientations were measured within a single area of  $1.4\text{ mm} \times 1.4\text{ mm}$  using an image analysis system (IMCO-1000, Kontron Elektronik GmbH, Munich, Germany) with a full color CCD camera. The area was digitized with a resolution of  $512 \times 512$  pixels and displayed on a monitor at a magnification of  $100\times$ . We drew lines with the orientation of the underlying cells on an acetate overlay taped to the screen monitor. This manual step was used because automated methods could not accurately identify individual cells and remove cells without a distinct orientation. The acetate tracings were then digitized using the image analysis system at a resolution of  $1024 \times 1024$ . The resulting images were analyzed to extract the orientation of each line. Briefly, the steps used were: thresholding to create a binary image where each pixel was either white or black, thinning to obtain single pixel wide lines, identification of individual lines, removal of background noise by ignoring lines too short to represent a cell's orientation, determination of the horizontal and vertical projection of each line, and finally, computation of the each lines orientation from its projections. The method's accuracy was verified by analyzing several different known distributions of lines generated by a computer graphics program. The discrete orientation probability distribution was determined from the individual cell alignments by counting the number of the cells within each  $5^\circ$  interval and dividing by the total number of cells. Cells oriented on both sides of the stretch direction (e.g.  $\pm 30^\circ$ ) were counted in the same interval.

We carefully considered the way by which we identified the orientation of each cell. Most cells were highly elongated and bipolar in shape with a well defined orientation. A small percentage, 5–10%, were clearly not oriented and were not sampled. However, some cells, although elongated, were not bipolar so that no single cell orientation was obvious. For these cells we used the orientation of the cell nucleus when it was identifiable. Otherwise we choose an orientation approximately midway between the two possible limits for the cell orientation. An additional error was probably introduced by the process of first drawing a line with each cell's orientation on an acetate overlay and then determining the orientation of the line. This process was used because we were unable to get our image analysis software to automatically distinguish individual cells and determine their orientation from the original image. To verify that our methods of determining cell orientation would not invalidate our conclusions, we first tested our method on a known population of simulated cells (short straight lines) drawn by a computer graphics program. After collecting our data, we also studied the effect of adding a random error to the measured cell orientations. The mean error was assumed zero and the standard deviation was taken as  $5^\circ$ , which is one-fourth the maximum range of orientations that might be assigned to a typical non-bipolar cell. Both studies showed that the likely errors introduced by our procedures would not alter our conclusions.

## Results

The extent to which the cell population became oriented away from the stretch direction depended on the magnitude of the applied cyclic stretch for both cell types (Figs. 3 and 4). The orientation distribution of cells grown on unstretched culture dishes was not different ( $P = 0.19$  for fibroblasts and  $P = 0.21$  for osteoblasts) from a uniform random distribution. In contrast, cells grown on cyclically stretched culture dishes showed a reduction in the percent of cells oriented at angles near the stretch direction,  $0^\circ$ , and an increase in the percent of cells oriented at angles near  $90^\circ$ . The increase in alignment was significant for 0–4% ( $P < 0.0001$ ), 4–8% ( $P < 0.00001$ ), and 8–12% ( $P < 0.0001$ ) for the fibroblasts, but only from 0–4% ( $P < 0.0001$ ) and 4–8% ( $P < 0.0001$ ) for the osteoblasts. We did not find an increase ( $P = 0.18$ ) in osteoblasts oriented away from the stretch direction when the stretch was increased from 8–12%. In no case did the cells assume a single orientation or even a narrow range of orientations. The fibroblasts had a more pronounced response to the stretch stimulus than the osteoblasts. At each level of stretch, fewer fibroblasts were oriented in the stretch direction and more were oriented at higher angles ( $P < 0.0001$ ).

Cell frequency (the probability of finding a cell) in each direction depended on the axial strain in that direction. The relation between cell frequency and axial strain did not depend on the applied substrate stretch magnitude (Fig. 5), although both cell frequency and axial strain depended on the applied stretch. Both types of cells exhibited axial strain limits above which few cells

were found. The strain limit was obtained by performing a linear regression and extrapolating the regression curve to zero cell frequency. Fibroblasts had a smaller strain limit,  $4.2 \pm 0.4\%$  (mean  $\pm$  95% confidence limit), than osteoblasts,  $6.4 \pm 0.6\%$ . No simple correlation was found between cell frequency and either transverse strain or shear strain. In fact, in both cases the relationship was multi-valued, with some levels of strain associated with both high and low cell frequencies (Fig. 6).

The extent of the cell realignment was near equilibrium at 24 h. No significant difference was found between 24 and 48 h ( $P = 0.12$ ) or between 48 and 72 h ( $P = 0.76$ ). However, only a small difference of questionable biological significance was found between 24 and 72 h ( $P = 0.027$ ).

## Discussion

Our major finding was that fibroblasts and osteoblasts became oriented in response to the axial strain, i.e. the substrate strain that changed the cell's length. While we have not completely ruled out the possibility that the cells also aligned in response to the transverse and shear strains, our data show these strains had a much smaller influence than the axial strain. This was most easily seen for shear strain, which was symmetric about the  $45^\circ$  direction (Fig. 2). The cell frequency distributions, however, were not symmetric, but skewed away from the stretch direction (Fig. 4(a), (b)). The symmetry of the shear strain and lack of symmetry of the frequency distributions explain the shape of the cell frequency

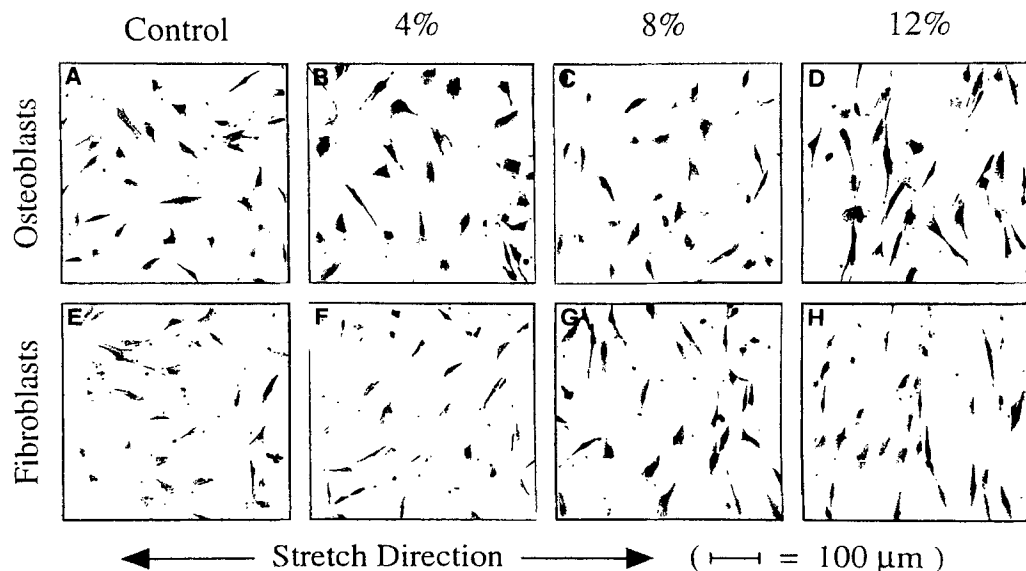


Fig. 3. Composite photomicrographs showing fibroblast, bottom row, and osteoblasts, top row, stained cultures after 24 h of cyclic stretch. The cells in the control cultures, left column, have no apparent preferential orientation. The 4% stretch clearly reduced the number of cells oriented in the stretch direction (horizontal). This effect was increased at 8% and 12% stretches.

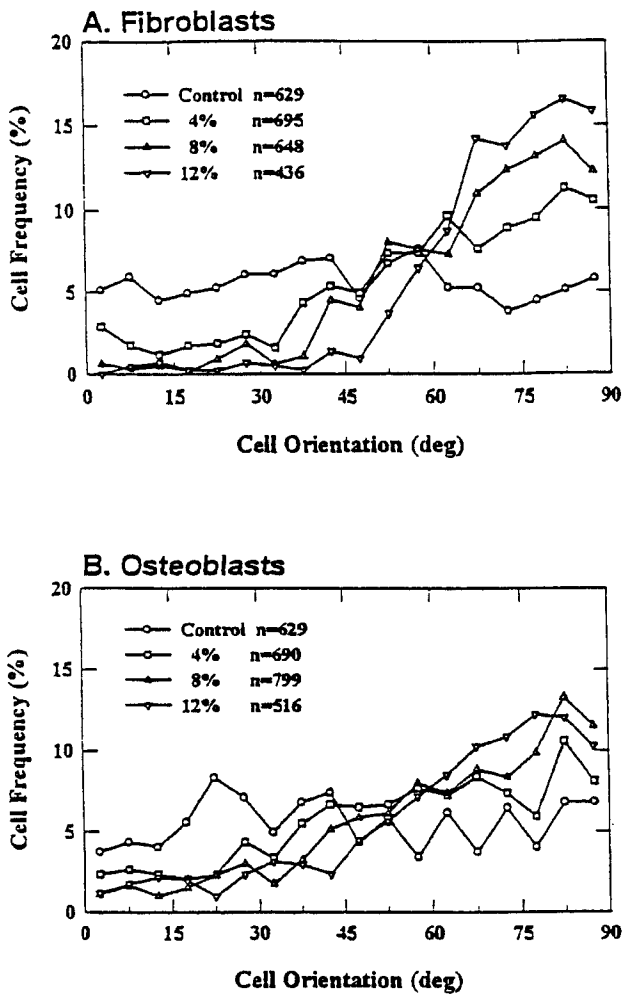


Fig. 4. Cell frequency depends upon orientation relative to the stretch direction (0°) for both cell type, (a) fibroblasts and (b) osteoblasts and for different stretch amplitudes. The distributions for the control cultures were not significantly different from random cell alignment where an equal number of cells (5.56% of the total) are expected for each interval. As the stretch magnitude increased, the percentage of cells oriented near the stretch direction decreased. Note that the frequency of cells near 60° does not depend on the applied stretch. This is the only direction where the axial strain is not affected by changing the applied stretch magnitude.

versus shear strain curves (Fig. 6(b)). The low shear strains that occurred near the stretch direction, 0°, were associated with low cell frequencies while similar low shear strains near 90° were associated with high cell frequencies.

It was less obvious to exclude the transverse strain as an important influence. Unlike shear strain, both the transverse and axial strains changed monotonically with increasing alignment away from the stretch direction: the transverse strain increased monotonically while the axial strain decreased monotonically (Fig. 2). Thus, either of these strains could potentially explain the skewed cell distributions we found. The choice of axial strain as the primary signal was based upon the studies con-

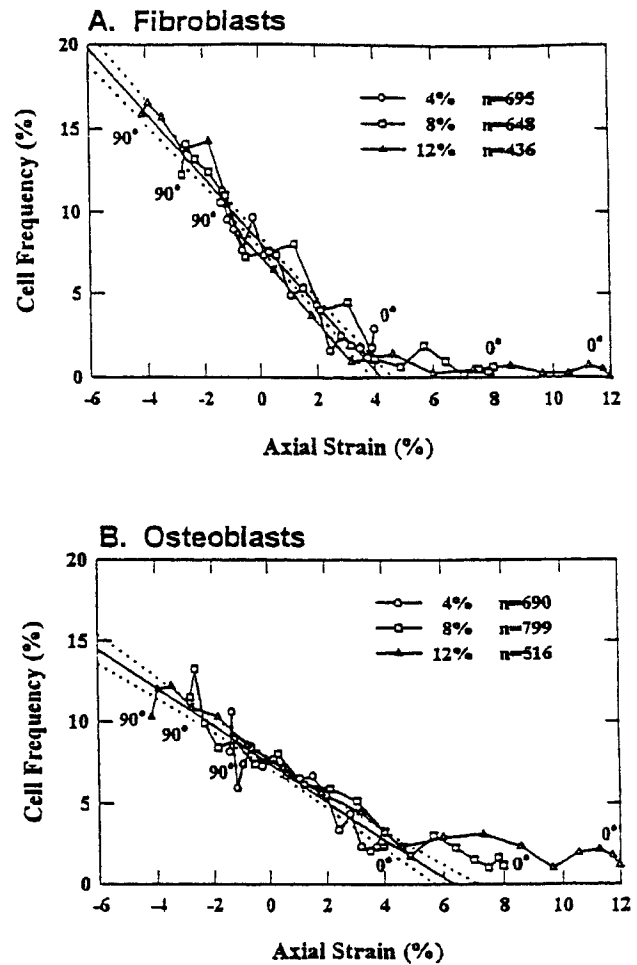


Fig. 5. The cell orientation frequency distributions had the same relationship to the axial strain, regardless of the applied stretch, for both types of cells. The datum points represent the cell frequency within each of the 5° angle intervals between the stretch direction (0°) and its perpendicular (90°). Points for cells oriented within 5° of the stretch direction are labeled 0° and those oriented within 5° of the perpendicular to the stretch direction are labeled 90°. Both types of cells also exhibited axial strain limits above which few cells were found. The limits were estimated by performing a linear regression analysis on the descending portion of the curves and extrapolating the fitted curve (solid line) to the zero frequency axis. The dotted lines show 95% confidence limits for the regressions.

ducted at different stretch magnitudes. There is but a single value of cell frequency at a given axial strain, regardless of the applied stretch magnitude (Fig. 5). In contrast, the relationship between cell frequency and transverse strain, like shear strain, was multi-valued with some values of transverse strain being associated with both low and high cell frequencies (Fig. 6).

Our finding that axial strain was the primary mechanical signal supports Buc's hypothesis that fibroblast alignment is an "... avoidance reaction to stretching based on cell's adhesion by linear focal contacts, which run parallel to the long axis of the cells, and which are associated with microfilaments." A number of investigators have now shown that cytoskeletal

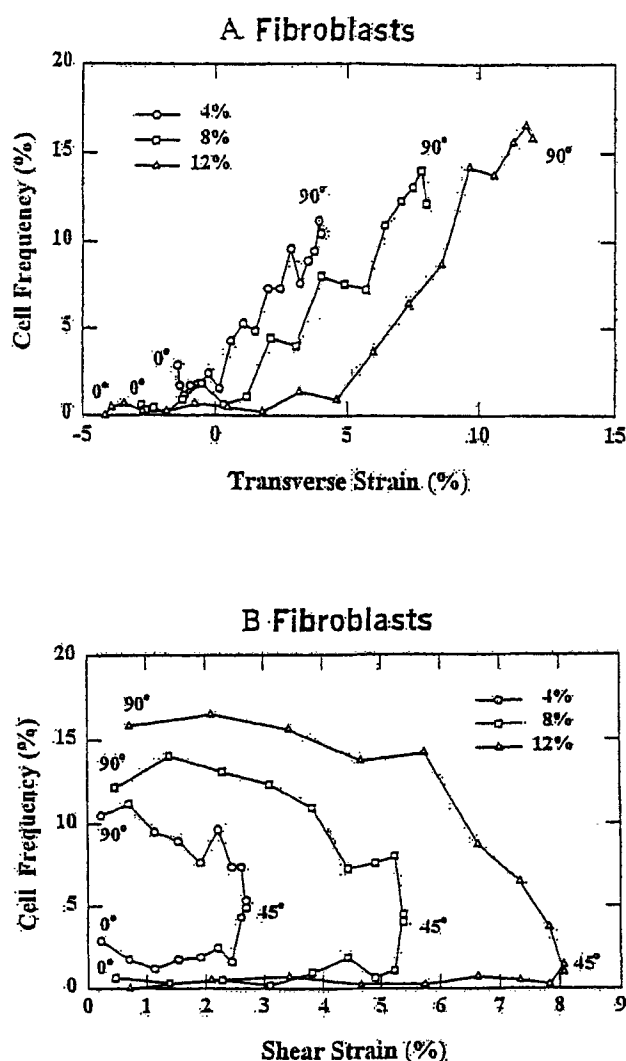


Fig. 6. The relationship between cell orientation frequency and the transverse strain (a) and shear strain (b) for the fibroblasts. The datum points for cells oriented near the stretch direction are labeled  $0^\circ$  while points near the perpendicular are labeled  $90^\circ$ . The relationships are multivalued: for some strain amplitudes there are both low and high cell frequencies.

reorganization begins prior to cell reorientation and that drugs, like forskolin and cytochalasin B, that disrupt the actin cytoskeleton also inhibit the orientation process [15,25,27]. It should be noted that there might be several mechanisms that can produce cell orientation as cells orient in response to electric fields, and variations in the elastic modulus of the substrata [9,13,18].

The cell alignment response appears to require both a direction-dependent (anisotropic) strain field and a direction dependence in the cell's ability to sense shape changes. The anisotropic strain field is required because the cell must be able to detect some magnitude difference in its deformation in differing directions if it is to favor some orientations over others. If in changing direction, each part of the cell is always exposed to the same strain

field, then no change will occur within the cell to make it prefer a new direction. Thus, an isotropic strain field provides no directional signal for the cell to detect.

The requirement of a direction-dependent sensing mechanism within the cell is slightly more difficult to deduce. Consider what would happen to a cell equally able to detect a deformation no matter what its direction is relative to the cell, i.e. the cell's sensing mechanism is isotropic. Imagine, now, a portion of the cell in the shape of a cube sitting in an anisotropic strain field. The deformations of the cube induce some set of signals within the cube. If the cube is rotated  $90^\circ$ , so the direction of two face pairs are interchanged, the signals induced within the cube by the strain field remain unchanged. This is because the strain field has not changed, and the sensitivity of the cell to detecting the strain in each direction is unchanged. Without a change in signal, there is no reason for this portion of the cell to change orientation. The same is also true for the rest of the cell, no matter what the cell's shape. On the other hand, if the cell's ability to detect strain were direction-dependent, a reorientation that placed its least sensitive sensing direction in the direction of highest strain would decrease in the signal induced within the cell. Thus, one of the most likely ways by which the cell can detect a change is that the ability of the cell's sensing deformation is also direction-dependent. The actin cytoskeleton may be the part of the cell's deformation sensing mechanism through its connection to integrin receptors that bind to the extracellular matrix [16]. The primary role of axial strain in determining the cell's orientation response probably results from the primarily axial cytoskeleton orientation, which develops in may stretched cells.

The orientation response does not appear to be the result of the cells attempt to minimize their axial strain as no preferential alignment was found along the zero axial strain direction of approximately  $60^\circ$  (Fig. 4(a), (b)). The finding that cells do not align in the exact zero strain direction is consistent with Dartsch's finding that a stretch threshold exists below which cell alignment does not occur [8]. The threshold was between 2% and 3.5% for smooth muscle cells. We found that neither osteoblasts nor fibroblasts aligned with 1% stretches while both exhibited some alignment with 4% stretches.

The extent of cell alignment, for stretches that exceed the threshold, depends on the applied stretch magnitude. The frequency distributions became significantly more skewed away from the stretch direction when the applied stretch was increased from 4% to 8% and again when it was increased from 8% to 12% (fibroblasts only). A similar result was previously found by Dartsch et al. [7,8] for both smooth muscle cells and endothelial cells grown to confluence.

An important finding, related to the dose-dependent response, is that an axial strain limit exists, above which

few cells are found. The strain limit can be estimated by extrapolating the cell frequency vs axial strain curves to zero cell frequency (Fig. 5). It is possible that Dartsch's threshold, which describes the lowest strain that initiates alignment, and our strain limit, which is the largest strain cells tolerate, are the two ends of a single process. Surprisingly, we found the strain limit for fibroblasts (4.2%) is lower than the strain limit for osteoblasts (6.4%), although fibroblasts are likely subjected to higher strains *in vivo*. We speculate that this result is due to a better-aligned and denser cytoskeleton in fibroblasts. Harris et al. [12] noted that traction forces produced by fibroblasts are much larger than needed for locomotion and speculated that "... morphogenetic rearrangement of extracellular matrices is the primary function of fibroblast traction and explains its excessive strength". The large traction force produced by the fibroblast reflects their highly oriented and dense actin cytoskeleton. We have already noted that disruption of the actin cytoskeleton inhibits the alignment response. Our data also suggest the reverse effect, that cells with highly oriented and dense cytoskeleton are less tolerant of stretches and align more readily.

Although a large number of different cell types orient in response to cyclic surface deformations, care must be taken in extrapolating our results to cells other than fibroblasts and osteoblasts. Our results also only apply to cells grown in low densities. Low cell densities were used to avoid the confounding effect produced by direct cell-cell contact. Cell-cell contact results in locally oriented groups of cells even in the absence of surface deformations. Clearly the orientation response of the cells within such a local group are not independent. Including cells from such a structure in a statistical sample would violate the independence criteria required for obtaining a simple random sample [24]. Therefore, cells from randomly selected regions on the culture dish were not sampled. We avoided regions where the cell density was high (cells in contact) and (relatively few cells), requiring the measurement of additional regions to obtain an adequate sample size for analysis. The sampling process used involved selecting four to six regions, each region typically containing 40–80 cells with few cell-cell contacts. All cells exhibiting a distinct orientation were sampled, except those having similar alignment to other cells they contacted. Such situations were treated as a single cell. While it is possible that the sampling scheme we used introduced some bias, it is unlikely this altered any of our conclusions as the bias would be present at all stretch levels.

In conclusion, the study suggests that the cell orientation response to cyclic culture dish deformation is primarily due to avoidance of large axial surface strains that induce change in the cell's length and that cells avoid directions where the axial strain exceeds a limit, which is specific to the cell type.

## Acknowledgements

This work was supported in part by grants from the Fulbright Commission (ESG), the National Institute of Arthritis, Musculoskeletal, and Skin Diseases (AR-21172) (ESG) and (AR-40411) (RAB), the Deutsche Akademischer Austauschdienst (RAB) and the Deutsche Forschungsgemeinschaft (CL77/2-1B) (CN-W). We wish to thank Mrs. Michaela Luderer for her technical assistance in the evaluation of the data.

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