Connective-tissue responses to defined biomaterial surfaces. II. Behavior of rat and mouse fibroblasts cultured on microgrooved substrates

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Abstract: Surface microgeometry strongly influences the shapes, orientations, and growth characteristics of cultured cells, but in-depth, quantitative studies of these effects are lacking. We investigated several contact guidance effects in cells within "dot" colonies of primary fibroblasts and in cultures of a transformed fibroblast cell line, employing titanium-coated, microgrooved polystyrene surfaces that we designed and produced. The aspect ratios, orientations, densities, and attachment areas of rat tendon fibroblasts (RTF) colony cells, in most cases, varied ($p < 0.01$) by microgroove dimension. We observed profoundly altered cell morphologies, reduced attachment areas, and reduced cell densities within colonies grown on microgrooved substrates, compared with cells of colonies grown on flat, control surfaces. 3T3 fibroblasts cultured on microgrooved surfaces demonstrated similarly altered morphologies. Fluorescence microscopy revealed that microgrooves alter the distribution and assembly of cytoskeletal and attachment proteins within these cells. These findings are consistent with previous results, and taken together with the results of our *in vivo* and cell colony growth studies, enable us to propose a unified hypothesis of how microgrooves induce contact guidance. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 85A: 326–335, 2008

Key words: microgroove; *in vitro*; cell-surface interactions; fibroblasts; cytoskeleton

INTRODUCTION

Cellular responses to substrate microgeometry. Soon after the advent of cell-culture technology, it became apparent that cultured cells characteristically respond to physical features of the substrates to which they are attached. Most normal mammalian cell types demonstrate attachment-dependence, substrate exploration, contact inhibition, and contact guidance.[1] Features of cellular dimensions (2–12 μm) are most effective at eliciting these responses.[2–6]

It is also apparent that contact guidance, through its effects on cell shape, regulates cell survival,[7–12] proliferation,[9,13,14] and differentiation.[15,16] These events are, of course, dependent on specific gene expression events,[9,17,18] substrate attachment,[19–23] and cell morphology[23–28] (for review, see Huang and Ingber[29]). Cell orientation, likewise, has profound effects on proliferation[4–6,30–32] and matrix organization.[33–35]

Contact guidance occurs on a variety of surfaces, including microgrooved titanium[36] and polymers,[37,38] and collagenous matrices.[39] Clark, et al.,[40] and others[4–6,31,41,42] showed that grooves of varying depths differentially affect cell orientation. The work of Chehroudi et al.,[37,38] Brunette,[43,44] and Ricci[6,45] demonstrates these effects both *in vitro* and *in vivo*. The influences of surface microgeometry on colonies of fibroblasts and bone marrow cells are evaluated in a companion report.[46]

Explanations of cellular contact guidance generally allude to characteristics of the cytoskeleton and its central role in cellular division, movement and attachment (for reviews, see Brunette, 1988; Walboomers and Jansen, 2001; Curtis, 2004).[47–49] The mechanisms that propose to explain contact guidance as observed on microgrooves and similar structures must include the roles of extracellular proteins, focal adhesions or adhesion plaques, cell-signaling cascades, and other elements, as well as

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the physical and chemical characteristics of the substrates. An extensive review of the literature revealed numerous reports of cellular and tissue-level responses to modified culture substrates, but few of those substrates employed features with average sizes of more than a few micrometers. Moreover, no previous reports describe the responses of cells to complex growth substrates while contained within a 3-dimensional matrix of collagenous or other proteins. We employed a novel, unique model to examine cellular interactions with surfaces having controlled, 3-dimensional substrate features of appreciable depth while surrounded by a 3-dimensional collagenous matrix. The cells we observed were simultaneously influenced by permanent, physical substrate features such as those that might be present on an implanted device, and by 3-dimensional cell-signaling events initiated such as those initiated by integrins and other cell-adhesion molecules interacting with extracellular matrix proteins.

Rationale

This study was undertaken as a corollary investigation to our examination of the growth tendencies of gel-dot colonies grown on microgrooved substrates.[46] The present study demonstrates that the attachment, morphometry, growth, migration, and orientation of fibroblastic cells are moderated by substrate microgeometry, both within a collagenous matrix and when grown as individual cells. The rationale underlying this study is that a simplified model of a connective tissue growing on oriented, microgrooved surfaces reveals alterations in cellular behavior that yield insight into the colonization of selected biomaterial implants by connective tissues. These investigations extend our studies, and those of other investigators, of the tissue-level effects of surface microgrooves on connective tissues and cell colonies[46] to include observations of the constituent cells within those colonies. However, while our previous results, and those of other investigators, demonstrate certain collective effects on populations of cells, they do not directly or quantitatively describe the behavior of individual cells within those tissues or cell colonies. We also employed a well-studied fibroblastic cell line to verify that our substrates were capable of orienting cells, directing their substrate-attachment tendencies, and organizing their constituents in a fashion that is consistent with the results of other investigators who examined cells grown directly on microgrooved or otherwise modified surfaces.

We measured the lengths, widths, projected attachment areas, densities and orientations of rat tendon fibroblasts (RTF) growing within collagen gel "dot" colonies on control and microgrooved substrates. The lengths, widths, aspect ratios, and attachment areas of individual cells reveal that microgroove dimension affected the sizes of cells and their abilities to spread. Cell densities reflect the rates of cell proliferation that occurred on each type of substrate. Cell orientations, determined by measuring the angular deviation of the direction of the long axis of each cell from the direction of the underlying microgrooves, indicate the ability of the various microgroove dimensions to direct the organization of the cells' contents.

MATERIALS AND METHODS

Cell culture substrate fabrication and analysis

We molded polystyrene substrates from silicon-wafer templates by a previously reported protocol.[46] Briefly, microgrooved silicon-wafer templates having parallel, square-wave microgrooves with periodicities of 3.55, 12.83, 15.75, and 23.75 μm were created with computer-aided design and multiple reactive-ion etching techniques. The finished templates were washed in hydrochloric acid and attached to three-inch aluminum rods with epoxy to provide rigidity. An example of a template surface is shown in Figure 1. Cell culture substrates were created from the templates by solvent-casting polystyrene (15 g in 100 mL ethyl acetate) under a vacuum. The solvent was evaporated for 24 h, and after a 5-h water wash, the replicates were peeled from their respective templates and cut into 15 or 50 mm circles. After air-drying, the substrates were coated with titanium oxide, affixed within 24-well tissue culture plates or 60-mm Petri dishes, and ethanol-sterilized. The dimensions of the substrate microgrooves were measured by transmission light microscopy and scanning

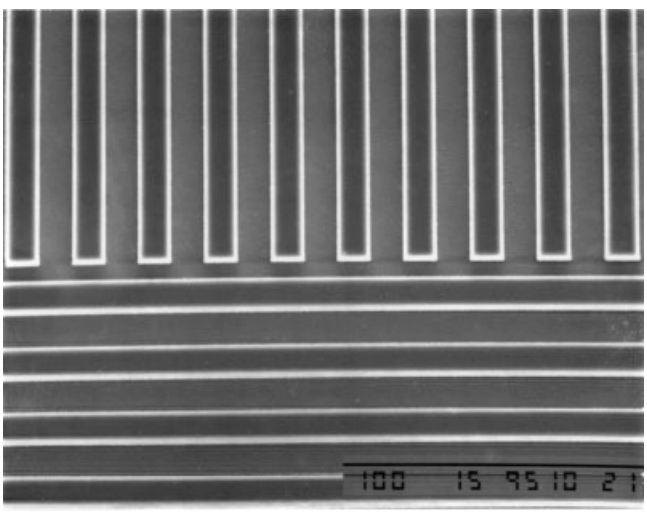


Figure 1. Scanning electron micrograph of a portion of a silicon-wafer mold bearing 12-μm microgrooves. Bar = 100 μm.

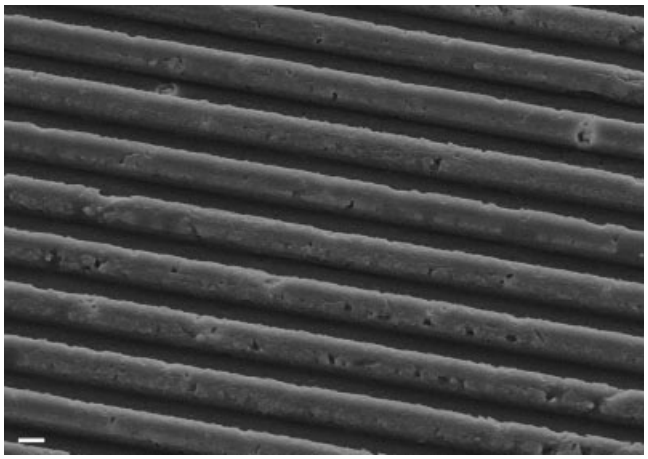


Figure 2. Scanning electron micrograph of a portion of a silicon-wafer mold bearing 12-μm microgrooves. The image shows the intersection of areas bearing microgrooves that intersect at a right angle. Bar = 100 μm.

electron microscopy, as described previously. An example of a microgrooved, polystyrene cell culture substrate is shown in Figure 2. The detailed features of our culture substrates are described in Table I.

Preparation of rat tendon fibroblast (RTF) cultures

RTF cultures were initiated from explanted hindfoot extensor tendons of male, 14 day-old Sprague–Dawley rats, as previously described.[46] In brief, the rats were sacrificed and the tendons were removed and used to establish explant cultures after the sheath tissue was removed. The tendon explants were removed from the cultures after 12–17 days and the adherent cells were subcultured. RTF cells obtained this way were used to inoculate additional culture plates and to initiate dot cultures (see below). All dissection and culture procedures were conducted under standard, sterile conditions with reagents obtained from GIBCO/Invitrogen.

Establishment and growth of RTF "dot" colonies

Colonies were established by embedding RTF cells in a collagen gel by the method we have previously reported.[46] Briefly, 1st-, 2nd-, and 3rd-passage RTF cells were suspended in bovine type I collagen solution (Collagen Corporation) and dispensed in 2-μL droplets (containing 20,000 cells each) onto the control and 2, 6, or 12 μm-microgrooved substrates. The cell-matrix mixture thereafter polymerized to form dot colonies.

Collagen-gel dot colonies containing RTF cells were grown on substrates bearing microgrooves of each dimension detailed above, as well as on flat, control areas on the same substrates. RTF cells from the same animals were used to initiate dot colonies on the flat control and experimental sides of each 50 mm-diameter substrate fitted within a 60-mm culture plate. The cultures were maintained under standard sterile conditions in appropriate media.

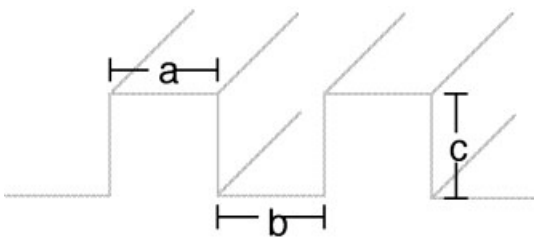
Assessment of cellular parameters within RTF colonies

RTF cells were grown on control (nongrooved) substrates and on each of the experimental substrates for 8 days. The cultures were then fixed in 10% phosphate-buffered formalin and stained with toluidine blue. Cultures were examined by light microscopy and representative colonies were photographed with an imaging system consisting of a Leitz Orthoplan transmission light microscope or Wild MC3 stereomicroscope, an MTI CCD72 video camera, an Apple Macintosh computer with Perceptics video acquisition hardware, and Scientific Imaging Solutions Workstation TCL-Image image processing/image analysis software. We randomly selected 6–7 dense, nonconfluent, internal areas located halfway along the radius of each colony, for analysis. Cell density, but not cell size or orientation, differed by site within the colonies. Cells at the growing edges of the colonies were sparser than at the assay point, while cells within the original dot were too dense to characterize individually. This method established a systematic means of selecting areas containing individual cells that formed an incomplete monolayer and were free of extensive cell–cell contact. We captured digital images of these areas, and then measured the lengths (in μm), widths (in μm), aspect ratios (length-to-width ratios, also known as eccentricity), attachment areas (in μm^2), densities (in cells/mm^2), and orientations (angular deviation, in Δ°) of individual cells within the selected areas. For control colonies, the horizontal axes of the captured images were used as reference lines for determining cell orientations.

Cytological assessment of fibroblasts cultured on microgrooves substrates

NIH-3T3 fibroblasts were obtained from American Type Culture Collection and maintained under standard, sterile conditions with reagents obtained from GIBCO/Invitrogen. Suspensions of 4000 first-second- and third-passage NIH-3T3 cells were seeded onto control and 2, 8, or 12 μm-microgrooved substrates and maintained in culture for 4 or 8

TABLE I
Schematic Diagram and Chart Detailing Substrate Microgroove Dimensions



Template Name	Microgroove Dimensions (a × b × c) νm³	Microgroove Periodicity (νm)
2	1.80 × 1.75 × 1.75	3.55
6	6.50 × 6.33 × 5.00	12.83
8	8.00 × 7.75 × 7.50	15.75
12	12.00 × 11.75 × 7.50	23.75

days. At those times, the substrates were removed and fixed in glutaraldehye for SEM observation or in paraformaldehyde for rhodamine–phalloidin, antivinculin, or fluorescein–antiphosphotyrosine staining. Cultures stained with antivinculin were subsequently stained with a rhodamine-labeled secondary antibody. Fluorescent probe-labeled cultures were examined by epifluorescence microscopy (Leitz Orthoplan), and representative areas were photographed. Rhodamine–phalloidin was obtained from Molecular Probes and antibodies were obtained from Sigma Chemical.

Data analysis and statistics

Data was recorded in MicroSoft Excel spreadsheets for statistical analysis. The mean (μ), standard deviation (SD), and sample size (n) were calculated for each group (i.e., microgroove dimension). The means of all groups within an experiment were compared by one-way analysis of variance (ANOVA). The means of individual groups were compared directly with Student's t-test (2-tailed, unpaired) for nonparametric data. Graphs were prepared from the data spreadsheets with Kaleida Graph (Synergy Software). Group means, with corresponding SD and n values are indicated, as are levels of statistical significance, if any. In each graph, the level of statistical significance for the mean of each group vs. any other group mean(s) is indicated by the presence of the group name in standard type (i.e, C, 2, 6, 8, or 12) for p-values < 0.05 and in boldface type for p-values < 0.01 (i.e, **C**, **2**, **6**, **8** or **12**).

RESULTS

RTF cell morphology

All three microgrooved surfaces had pronounced effects on the aspect ratios, attachment areas, densities, and orientations of individual RTF cells grown in gel dot colonies, relative to controls. Growth on the microgrooved surfaces increased RTF cell aspect ratios, reduced cell spreading and cell densities, when compared with controls, and effectively oriented the cells in parallel with the microgrooves on the surface. These results correlate with the tendencies of cell colonies grown on microgrooved surfaces to be smaller, elongated and oriented, compared to controls.[46]

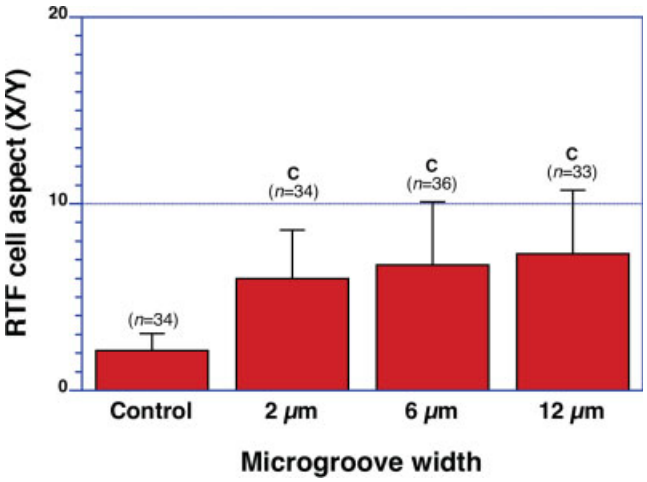


Figure 3. Mean aspect ratios of RTF cells grown on control and microgrooved substrates. Notations report SD, sample size and levels of statistical significance in all graphs, as described above. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

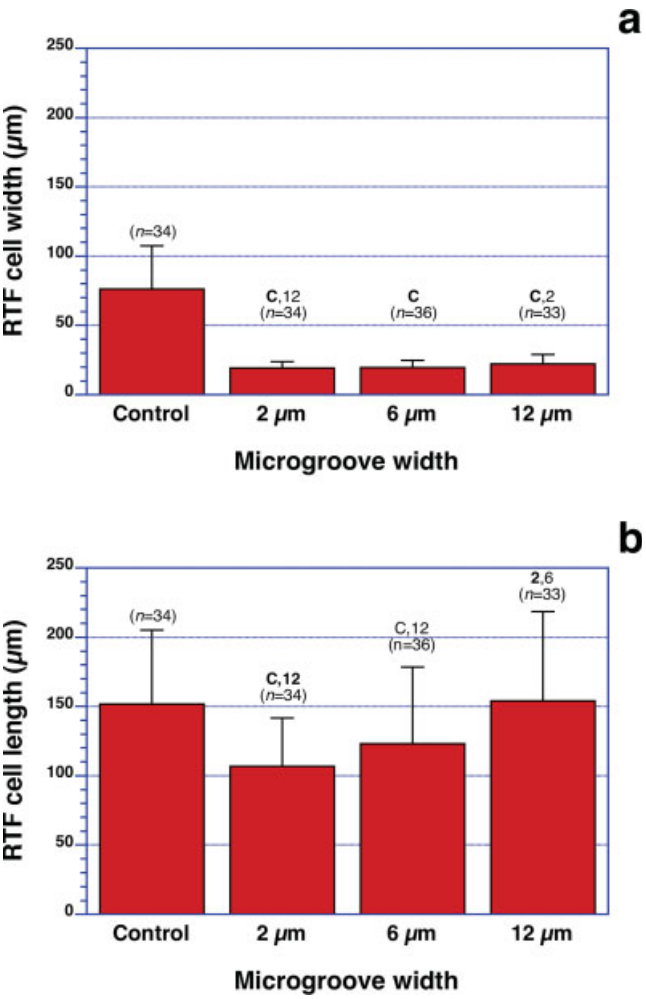


Figure 4. Mean widths (a) and lengths (b) of RTF cells grown on control and microgrooved substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Our experimental design revealed significant variance among mean group values of RTF cell lengths, widths, areas, aspect ratios, orientation, and density after 8 days in culture. In all cases, the confidence levels for determining statistical significance (p-value) were 99% or greater (i.e., $p \leq 0.01$); in many cases our results carried extremely high significance levels (i.e., $p \leq 10^{-32}$).

The mean aspect ratio of RTF cells grown on each microgrooved surface was significantly greater than

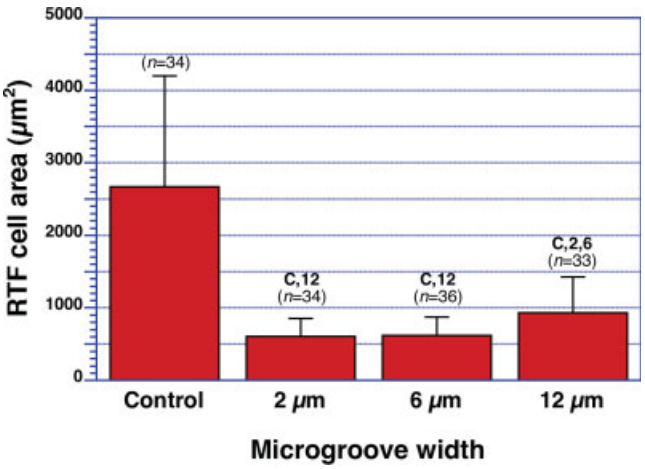


Figure 5. Mean attachment areas of RTF cells grown on control and microgrooved substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cells grown on the control surfaces (Fig. 3). This is chiefly due to significantly decreased widths of cells grown on microgrooved substrates, indicating that cell spreading was inhibited across the microgrooves [Fig. 4(a)]. Cell length, on the other hand, was variably affected by the microgrooves, with only 2- and 6-μm grooves significantly impairing elongation of cells. The mean length of cells cultured on 12-μm microgrooves was slightly, but not significantly, increased over that of control cells [Fig. 4(b)].

Mean cell attachment area was significantly decreased in cultures grown on microgrooves, when compared with cells grown on control surfaces (Fig. 5). These results describe projected attachment areas and not total attachment areas, since vertical surfaces are neither measured nor included in our calculations.

Mean cell density was significantly decreased in cultures grown on microgrooves, when compared with cells grown on control surfaces (Fig. 6). Again, these data represent projected substrate areas and not total surface areas.

Microgrooves are powerful determinants of cell orientation *in vitro*. Cellular orientation, expressed as degrees of deviation from the direction of microgroove direction, was profoundly and significantly reduced in cultures grown on microgrooves, compared with the high deviation from horizontal seen in cultures grown on flat, control surfaces (Fig. 7). Predictably, the nonoriented control cultures had mean angular deviations of nearly 45° and a high SD.

3T3 fibroblast cytology

By day 4, NIH-3T3 fibroblasts had adhered to all substrates, and by day 8, showed considerable growth, which approached confluence in places. Cells grown on microgrooved substrates were nearly uniformly oriented in parallel with the direction of the microgrooves (Figs. 8–10). There was no predominant orientation or shape in cells grown on control surfaces [Fig. 9(a)]. Cells cultured on 2- and 8-μm grooves mostly grew atop the ridges, apparently bridging the grooves between ridges. Cells cultured on 12-μm grooves mostly grew either atop the ridges or within the grooves, and less infrequently bridged the grooves

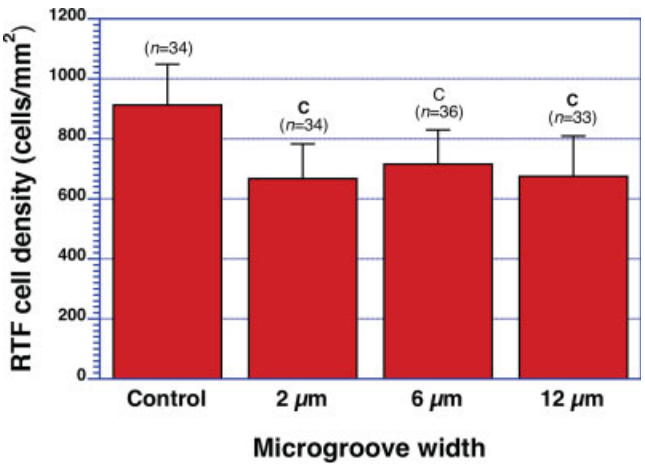


Figure 6. Mean densities of RTF cells grown on control and microgrooved substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

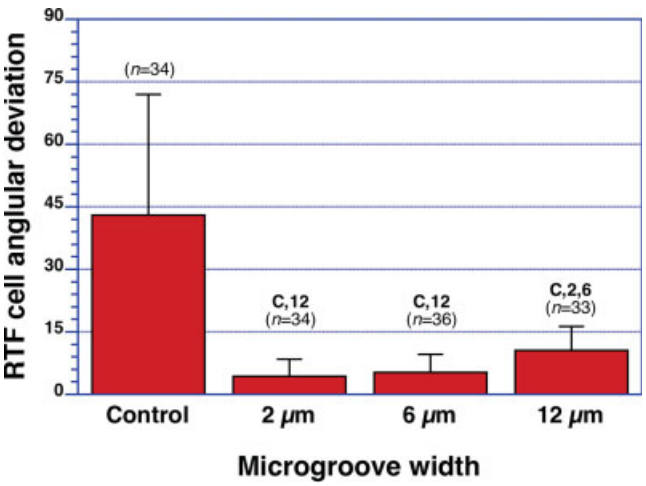


Figure 7. Mean orientations of RTF cells grown on control and microgrooved substrates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

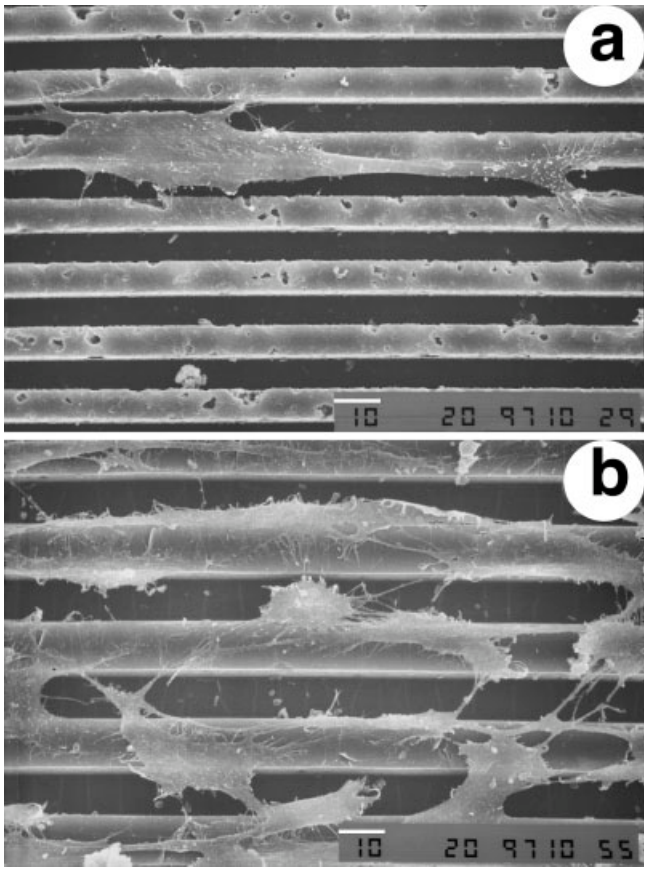


Figure 8. Scanning-electron micrographs of NIH-3T3 fibroblasts grown on 8- (a) and 12-μm (b) microgrooved substrates for 8 days. The individual cell grown on 8-μm grooves is attached to several adjacent ridges, bridging the intervening grooves. The cells grown on 12-μm grooves most frequently lie atop the ridges or within the grooves. Both microgroove dimensions impose a strong orientation effect upon the cells. Bar = 10 μm.

between ridges. These results agree with observations of RTF cells grown on similar substrates.

Stress fibers (microfilaments) were evident in control cultures of NIH-3T3 fibroblasts after 4 days [Fig. 9(a)] by rhodamine–phalloidin staining. The cytoplasms of cells cultured on microgrooved substrates showed only diffuse evidence stress fibers after 4 days. After 8 days in culture, cells grown on 2- and 8-μm microgrooves demonstrated limited evidence of stress fibers [Fig. 9(b)], but cells grown on 12-μm microgrooves revealed no evidence of stress fibers [Fig. 9(c)]. The reduced optical clarity of microgrooved substrates does not explain their limitation of microfilament staining, since the epiillumination microscopy was employed in this assay.

After 8 days, focal adhesions and phosphotyrosine appeared to be limited to areas of cell-substrate contact. Basal cell membrane in contact with microgroove ridges demonstrated intense staining with antivinculin and antiphosphotyrosine, while antivinculin and antiphosphotyrosine staining was less intense in basal cell membrane overlying microgroove troughs (Fig. 10).

DISCUSSION

Cellular responses to substrate microgeometry

In this study, which was undertaken as a corollary investigation to our examination of the growth tendencies of gel-dot colonies grown on microgrooved substrates,[46] sought to quantitatively describe the responses of constituent cells to the substrate features. Our data suggest that the cells cultured in collagen gel dot colonies on the microgrooved surfaces were well aligned, but lower in density, less spread, and longer and narrower in shape than those grown on the control surfaces. These observations are in general agreement with those of other investigators.[3,15,24,32-35,40-43,50-63] The *in vitro* effects of microgrooved substrates are probably based on relationships between microgroove dimensions and the abilities of cells to attach, spread, and migrate. Interestingly, we observed the same general tendencies in these cells as occurred in their surrounding colonies, suggesting a hierarchical relationship between the colonies and their constituent cells. Control colonies and cells were densest, largest and round, while colonies grown on microgrooved substrates and the cells therein were sparser, smaller and elongated.[46]

Our results suggest an additional mechanism by which suppression of cell spreading may inhibit mitosis and promote directional cell migration. Folkman and Moscona were the first to show a direct relationship between cell spreading and proliferation *in vitro* based on modification of culture surface chemistry (Reviewed in Ricci et al.,[46]).[13] We demonstrate that the inhibition of cell spreading through the use of controlled substrate microstructure apparently causes a similar effect. Additionally, our results suggest that microgrooves encourage mitosis in the direction parallel to the microgrooves and discourage mitosis in the direction perpendicular to the microgrooves. The effect of microgrooved surfaces on cell attachment, shape, and orientation may explain our observations of altered colony growth on similar surfaces.[6,46] Our results are also in agreement with observations that microgrooves depths greater than 2 μm are required to control cell and tissue behaviors other than orientation.[41,42,64-66]

Our data shows that both cell spreading and cell density were lower on microgrooved substrata than on flat control surfaces. The cell attachment area data does not account for possible cell attachment on the vertical microgroove surfaces, since our light mi-

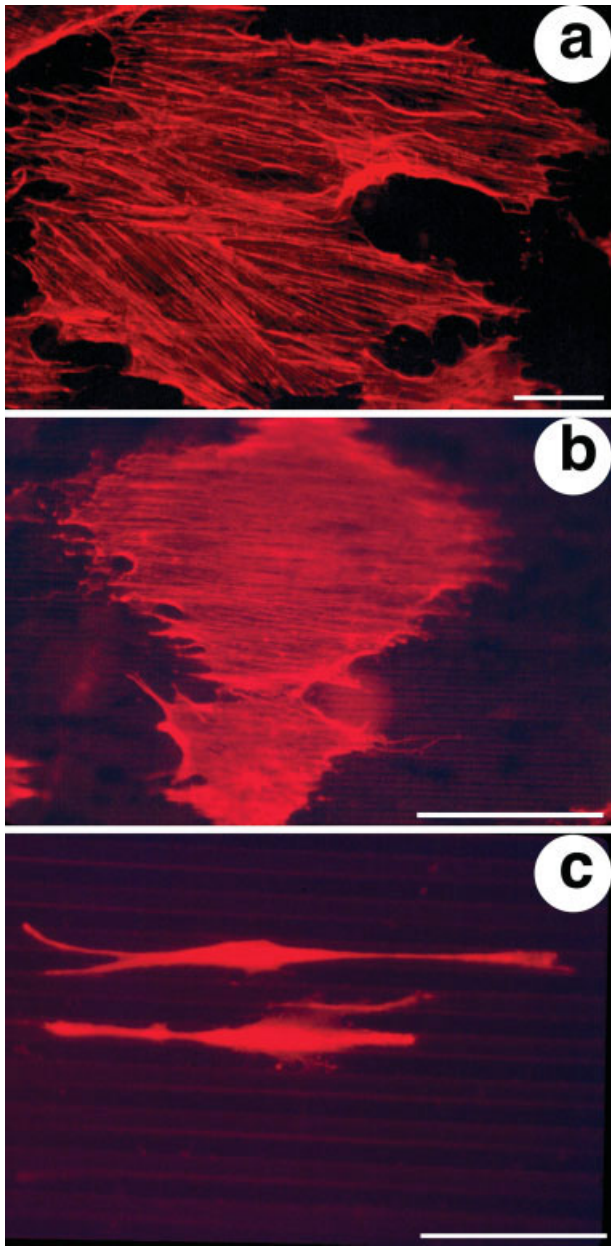


Figure 9. Fluorescence micrographs of rhodamine–phalloidin-stained NIH-3T3 fibroblasts grown on a control substrates for 4 days (a), and on 2-μm (b) and 12-μm (c) microgrooved substrates for 8 days. Cells grown on control surfaces lacked apparent orientation and shape, while those on microgrooves were oriented by the microgrooves. Stress fibers were evident in cells grown on control surfaces after 4 days, but became evident in cells grown on 2- and 8-μm grooves only after 8 days. Cells grown on 12-μm grooves had the most profoundly altered shapes and revealed no evidence of stress fibers after 8 days in culture. Bar = 100 μm (a, b, and c).

croscopic measurements were based on projection areas. However, it is unlikely that accounting for vertical-wall attachment would bring cell attachment areas to the levels seen on control surfaces, even if the extent of vertical-wall attachment were extensive. Our data also suggests that cell density is reduced by surface microgrooves, even though this conclusion is based on projected surface area measurements and not on the total actual surface areas of the microgrooved surfaces. Since square-wave microgrooved surfaces have, by definition, twice the surface areas of flat surfaces, our cell density data, if based on total surface area, is approximately one-half of our measured values. Taken together, the cell spreading and density data suggest that the cells grown on microgrooved surfaces did not utilize the majority of the area available for attachment.

Cytological responses to substrate microgeometry

Our observations of NIH-3T3 fibroblasts orientation by substrate microgrooves agree with patterns of RTF

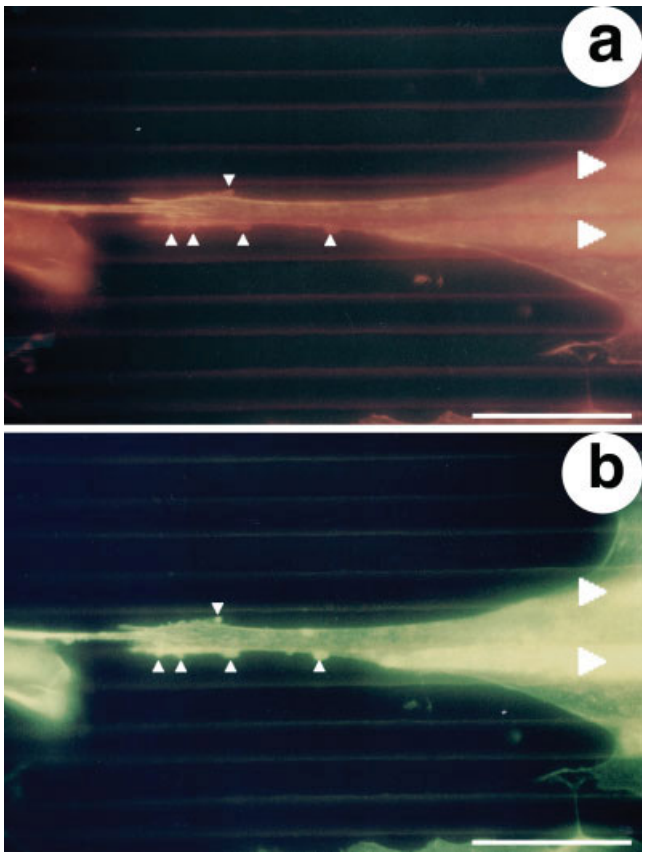


Figure 10. Fluorescence micrographs of dual-labeled NIH-3T3 fibroblasts grown on 12-μm microgrooved substrates for 8 days and stained for focal adhesions and associated cell signaling activity with fluorescent antibodies to vinculin (a) and phosphotyrosine (b). Areas of intense fluorescence due to antibody affinity correspond to areas of basal membrane of the cell body that are in contact with the elevated microgroove ridges (large arrowheads) and focal adhesions established by the lateral edges of the pseudopod (overlying a microgroove trough) where the pseudopod contacts the edges of neighboring ridges (small arrowheads). The optical complexity of the substrates reduces the resolution of these images. Bar = 50 μm (a and b).

cell growth on similar substrates, and with those of the investigators cited above. Moreover, the differential channeling of cells grown on microgrooves of varying dimensions agrees with similar observations among RTF cell growth on these substrates. Our finding of impaired stress fiber formation in NIH-3T3 fibroblasts cultivated on microgrooved substrates is intriguing, and differs from den Braber's observations of stress fibers within dermal fibroblasts after 3 days in culture.[55] When evident, stress fibers were aligned in parallel with the orientation of the NIH-3T3 fibroblasts and microgrooves, an observation that differs from den Braber's, but concurs with others'.[24,41,42] These discrepancies may have resulted from the different fibroblast phenotypes, substrate compositions, reagents or microscopy techniques employed. However, our observations of stress fibers in 4-day control cultures suggests that cell source or microgroove depth most likely caused these disparate results. The distribution of focal adhesions in our NIH-3T3 fibroblasts cultured on microgrooved substrates was in general agreement with these investigators' observations that focal adhesions were limited to areas of cell-substrate contact.[24,55,61]

Correlation with colony growth and *in vivo* studies

The present study complements our previous investigations of the influence of surface microgrooves on cell colony formation *in vitro*,[6] fibrous tissue and bone formation *in vivo*,[6,45] and osseointegration of dental implants.[67] Collectively, the results of these studies provide us with a broad base of information from which we hypothesize an explanation for the enhancement in osseointegration of microgrooved implants observed *in vivo*.[68] In brief, we suggest that microgrooves cause the orientation of connective tissue cells, in a fashion that is dependent on both cell type and microgroove dimension; this cellular orientation is imposed through directed establishment of focal adhesions and organization of the cytoskeletal; cellular orientation invokes the production of oriented matrix molecules[33-35,51,52,61]; the co-orientation of the extracellular and intracellular (cytoskeleton) matrix molecules supports the directional mitosis of the oriented cells[69] and the microgrooves guide cell migration[50] (resulting in co-oriented cell colonies[46]); the organized cells and matrix develop into an organized tissue, which in the case of an osseointegrated implant consists of bony trabeculae oriented preferentially with respect to the direction of mechanical forces applied to the tissue-implant interface[67,68]; the superior shear-resistance of oriented bone improves the quality and durability of osseointegration of an implant, resulting in a stabler, longer-lived implant.

Cellular responses to surface microgrooves lie at the heart of this hierarchical strategy for engineering the implant-interfacial tissue. This fact is elegantly demonstrated by the fact that the colonies of cells cultured on microgrooved substrates closely resemble the shapes of their constituent cells. Microgrooves 2–12 μm in width are capable of altering cell colony growth, with the strongest effects produced by grooves in the narrow size range of 4–8 μm. Perhaps the most important cellular characteristic in this regard is the "substrate exploration" function, a mode of behavior dependent on cell attachment, the cytoskeleton, cell shape, directional cell-signaling processes, and cell movement. Exploiting this behavior, surface microgeometry can dictate the formation of exploratory cell processes.

Our results support a stochastic model of contact guidance—the concept that microgrooves influence the probability of cell migration in predictable directions. We demonstrate that microgrooves of selected sizes can reduce the probability of cell migration perpendicular to microgroove orientation to nearly zero while increasing the probability of cell migration parallel to the microgrooves. This has the effect of stopping overall colony growth in one direction while stimulating colony growth in another direction. We also describe differential effects of microgrooves on two cell types, supporting the contention that cells of different sizes are similarly influenced by microgrooves of different dimensions.

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